Cross-Resistance and Collateral Susceptibility to Antifolic Antimalarial Compounds¹

CARL C. SMITH AND CLARA S. GENTHER

Department of Environmental Health and Christ Hospital Institute of Medical Research, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45219

Received for publication 1 May 1972

Series of strains of *Streptococcus faecium* ATCC 8043, *Lactobacillus casei* ATCC 7469, and *Pediococcus cerevisiae* ATCC 8081 with increasing resistance to the active antifolate antimalarial drugs chlorguanide triazine (CGT), pyrimethamine (PM), and trimethoprim (TMP) were isolated. These mutant strains, stable for at least 3 to 5 years, were examined for cross-resistance and collateral susceptibility to the above compounds and to methotrexate (MTX). Generally, they exhibited cross-resistance to all four compounds, but resistance of a strain to one compound did not predict accurately its resistance to another drug. Unexpectedly, *L. casei* resistant to CGT exhibited collateral susceptibility to MTX, TMP, and PM varying from 5- to 20-fold. *P. cerevisiae* developed resistance to CGT readily but maintained its susceptibility to PM and TMP after prolonged exposure to these compounds. Resistance to these antimalarial antifolates was accompanied by only low-grade cross-resistance to MTX, a representative antileukemic antifolate agent.

The emergence of strains of *Plasmodium falciparum* malaria parasites resistant to chloroquine, a drug long regarded as free from this liability, made it important to find new active agents. Folic acid antagonists, which constitute another type of antimalarial, particularly in combination with a sulfonamide, are active against some of these chloroquine-resistant strains (13, 15). Therefore, as part of a national program, we have been testing new synthetic compounds for antifolate activity using bacteria that require preformed folates for growth.

It is well recognized that malarial parasites frequently develop resistance to active antifolate agents (15, 20). Furthermore, because of the general structural similarities of these compounds, cross-resistance to other antifolates has been a frequent but not invariable finding both in malarial parasites (1, 13, 14, 16) and in bacteria (9, 17). The conclusion has been drawn that resistance to this type of compound is due to a multiplicity of mechanisms (8, 9).

With the hope of separating some of the factors determining antifolate resistance, we have isolated strains of folate-requiring bacteria at various time intervals during exposure of the cultures to chlorguanide triazine (CGT), the active me-

tabolite of chlorguanide, pyrimethamine (PM), and trimethoprim (TMP). The development of these strains and their patterns of cross-resistance and collateral susceptibility are the subject of this report. The resistant strains were also tested against methotrexate (MTX) to determine whether the pattern of response extends to folate analogues which are relatively inactive in malaria but are active in the therapy of leukemia.

MATERIALS AND METHODS

Test organisms. The organisms used included *Streptococcus faecium* var. *durans* (formerly classified as *Streptococcus faecalis*) ATCC 8043 (3), *Lactobacillus casei* ATCC 7469, and *Pediococcus cerevisiae* ATCC 8081. They were chosen because they have differing and well-defined folic acid requirements (11), and are susceptible to antifolate compounds. The parent susceptible strains were maintained by weekly transfer, the *S. faecium* strain on yeast extract-agar and the *L. casei* and *P. cerevisiae* strains on yeast extract-peptone-agar.

Test media. Standard folic acid assay media were prepared as described by Flynn et al. (4) with the exception that glucose, folic acid, and calcium leucovorin solutions were sterilized separately and added aseptically at the time of test. Amounts of 8 ml of the appropriate basal medium were placed in tubes and sterilized by autoclaving at 121 C for 10 min. Folic acid or calcium leucovorin diluted in 25% sterile glucose solution was added in 1-ml quantities. The final concentrations of folic acid were 0.002 µg/ml for *S. faecium* and 0.001 µg/ml for *L. casei*; the final

¹ Contribution no. 1069 from the Army Research Program on malaria. Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., 13-18 April 1969.

concentration of calcium leucovorin was 0.001 $\mu\text{g/ml}$ for *P. cerevisiae*. Stock solutions of PM in 0.04 N HCl and of CGT, TMP, and MTX in distilled water were sterilized by membrane filtration. Drug dilutions in 1 ml of distilled water were added to give a final volume of 10 ml.

Vitamin-free casein hydrolysate-acid (10%) suitable for folic acid assay and the purine and vitamin additives used to prepare the test media were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; folic acid and calcium leucovorin, from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; CGT (cycloguanil, 1-*p*-chlorophenyl-1,2-dihydro-2,2-dimethyl-4,6-diamino-s-triazine), from Parke, Davis & Co., Detroit, Mich.; PM (Daraprim, 2,4-diamino-5-*p*-chlorophenyl-6-ethyl-pyrimidine) and TMP (2,4-diamino-5-[3',4',5'-trimethoxybenzyl]-pyrimidine), from the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, Washington, D.C.; and MTX (amethopterin, 2,4-diamino-N¹⁰-methylpteroyl-glutamic acid) was supplied by James L. Rueggesser, Lederle Laboratories.

Growth conditions. The test inoculum was prepared as follows. The bacterial strains were transferred twice in test medium; the first transfer was incubated for 24 hr and the second for 16 to 18 hr at 37 C. The resulting culture was centrifuged and washed twice, and the suspension in 0.85% saline was diluted to give a reading of 50 Klett units (Klett-Summerson photoelectric colorimeter, filter 66). To each tube of test medium was added 0.1 ml of this suspension, providing an initial concentration of approximately 3.5×10^6 to 6.0×10^6 cells per ml as determined by plate count. The cultures were incubated at 37 C for 22 hr, and turbidity was recorded in Klett units.

Selection of resistant strains. Strains resistant to CGT, PM, or TMP were obtained by serial passage of the organisms through medium containing increasing concentrations of the compounds. At each transfer, a series of drug dilutions was employed that permitted growth ranging from heavy to very light. Control growth of the organisms under the conditions listed above produced Klett readings of 300 to 350 for *S. faecium* and *L. casei* and 200 to 250 for *P. cerevisiae*; these readings represented approximately 2×10^8 to 3×10^8 cells per ml for *S. faecium* and *P. cerevisiae* and 9×10^8 cells per ml for *L. casei* as determined by plate count. Subinoculations into drug-free medium were made from tubes showing approximately 50% of control growth. These cultures were incubated for 16 to 18 hr, washed, and resuspended in saline to prepare the standardized inocula for the next series of tubes. In this way, it was possible to determine accurately the concentration of compound needed to cause 50% growth inhibition (IC_{50}) of a strain at each passage.

Two or three transfers were made each week in drug-containing medium. On Friday of each week, the culture was passaged to yeast extract or yeast extract-peptone-agar (see above), incubated for 24 hr, and refrigerated over the weekend. This preparation was used as the source of inoculum at the beginning of the next week. Cultures selected at various intervals were retained. They have been designated by the compound

to which they were exposed and in order of isolation, e.g., CGT_r-A, CGT_r-B . . . CGT_r-H. These isolates were maintained by monthly transfer in drug-free agar and were used to determine cross-reactions to the other compounds.

RESULTS

The concentrations of CGT, PM, TMP, and MTX needed to produce 50% inhibition of growth of the parent susceptible strains of *S. faecium*, *L. casei*, and *P. cerevisiae* are given in Table 1. The relative activities of the compounds against these parent strains may be summarized as follows: *S. faecium*, MTX > PM > CGT > TMP; *L. casei*, MTX > TMP > PM > CGT; *P. cerevisiae*, MTX > PM > TMP > CGT. In each case, MTX was the most active compound. PM was always more active than CGT, whereas TMP changed position for each organism.

The patterns of response obtained when *S. faecium* was exposed to increasing concentrations of CGT, PM, or TMP are shown in Fig. 1. It can be seen that resistance to CGT developed very rapidly; thus, strain CGT_r-H isolated after only 26 passages was 192,000 times as resistant to CGT as the parent strain. The CGT-resistant isolates showed increased resistance to PM, but the ratios of CGT resistance to PM resistance were not in the same proportion for the different strains. Cross-resistance to TMP in the CGT-resistant strains was unexpectedly low, reaching only 16-fold. Resistance to MTX was even lower, being only sixfold.

The development of resistance of *S. faecium* to PM followed a very different pattern. Resistance developed slowly in a stepwise manner so that at the end of 74 passages the PM_r-F strain was 13,500 times as resistant as the parent strain. There was pronounced cross-resistance to CGT, but the ratio of CGT resistance to PM resistance for the PM_r-F strain was only 5 whereas the same ratio for CGT_r-H was 150. Resistance to TMP increased with PM resistance, but the maximal increase was only 150-fold. The most resistant strain, PM_r-F, showed a 33-fold increase in resistance to MTX.

Development of resistance of *S. faecium* to TMP resembled the pattern observed with PM in that there were stepwise increases in resistance followed by long intervals without change. The TMP resistance of TMP_r-G was 460 times that of the parent strain, whereas its CGT and PM resistance were 80- and 75-fold, respectively. The highest resistance to MTX in the TMP-resistant organisms was sevenfold.

The development of resistance by *L. casei* to CGT, PM, and TMP is depicted in Fig. 2. Re-

TABLE 1. Concentrations of chlorguanide triazine (CGT), pyrimethamine (PM), trimethoprim (TMP) and methotrexate (MTX) required to produce 50% growth inhibition (IC₅₀) of parent susceptible strains

Compound	IC ₅₀ (μg/ml) ^a		
	<i>S. faecium</i>	<i>L. casei</i>	<i>P. cerevisiae</i>
CGT.....	0.007 (0.0049-0.008)	5.1 (3.6-5.7)	10.1 (5.6-16.5)
PM.....	0.0022 (0.0015-0.0028)	1.26 (1.2-1.3)	1.1 (0.97-1.2)
TMP.....	0.024 (0.01-0.034)	0.054 (0.042-0.072)	3.4 (1.6-6.2)
MTX.....	0.00055 (0.0005-0.0006)	0.00002 (0.000016-0.000024)	0.125 (0.12-0.13)

^a The values given are the averages and associated ranges determined from 8 to 10 experiments carried out at intervals during a 5-year period.

FIG. 1. Development of patterns of resistance when *Streptococcus faecium* was exposed to increasing concentrations of chlorguanide triazine (CGT), pyrimethamine (PM), or trimethoprim (TMP).

FIG. 2. Development of patterns of resistance and collateral susceptibility when *Lactobacillus casei* was exposed to increasing concentrations of chlorguanide triazine (CGT), pyrimethamine (PM), or trimethoprim (TMP).

sistance to CGT developed easily. *L. casei* is much less susceptible to CGT than is *S. faecium*; thus, the fold increases in resistance were less although the IC_{50} of *L. casei* CGT_r-H was about the same as that of *S. faecium* CGT_r-H. The CGT-resistant *L. casei* strains retained their susceptibility to PM, TMP, and MTX until CGT_r-F, CGT_r-G, and CGT_r-H were reached. These latter strains, which showed a 150-fold increase in resistance to CGT, exhibited unexpected collateral susceptibility to the other compounds. The increase in susceptibility to PM was 20-fold, to TMP, 10-fold and to MTX, 5-fold.

In *L. casei*, a 40-fold increase in resistance to PM developed rapidly in the first few transfers followed by only small changes during 60 additional passages. These cultures exhibited parallel increases in resistance to CGT, TMP, and MTX.

Resistance to TMP in *L. casei* developed in two major steps, and there was concomitant development of cross-resistance to CGT and PM. The highest resistance obtained to MTX was 19-fold.

The development of resistance by *P. cerevisiae* to CGT is shown in Fig. 3. Resistance to CGT developed easily, but the increase in resistance (26-fold) was limited by the low susceptibility of the parent strain and the solubility of the drug in the medium. Only a three- to sixfold cross-resistance developed to PM, TMP, and MTX.

Although *P. cerevisiae* was transferred 65 times in medium containing PM and 40 times in TMP, no significant changes in susceptibility to either compound were detected (Fig. 3).

The resistant strains for all three organisms have been retested at approximately 6-month intervals. Their cross-resistance and collateral susceptibility patterns have not changed over a period of 3 to 5 years.

DISCUSSION

Few studies have been made of the development of resistance or of the cross-resistance patterns to antifolate antimetabolic drugs in bacteria. Foley and Watson (5) obtained strains of *S. faecium* resistant to D-54 HCl (4,6-diamino-1-[3',4'-dichlorophenyl]-1,2-dihydro-2,2-dimethyl-s-triazine HCl) which were cross-resistant to a number of dihydrotriazines but exhibited no cross-resistance to 2,4-diamino-pyrimidines (such as PM) or to aminopterin or MTX. Singer et al. (17) isolated strains of *L. casei* resistant to PM that were cross-resistant to all of the folic acid antagonists tested, including representative diaminopyrimidines, pyridopyrimidines, pteridines, and benzyl- and phenylpyrimidines. The same workers also reported that strains of *Proteus vulgaris* resistant to 2,4-diamino-5-(3',4'-dimethoxybenzyl)pyrimidine were cross-resistant to benzylphenoxy- and pyridopyrimidines; they were as susceptible as the wild strain to PM and a quinazoline.

Nichol (12) reported that a strain of *P. cerevisiae* selected for its ability to grow in folic acid medium was more susceptible than the parent strain to structural analogues of folic acid such as aminopterin and amethopterin but showed no change in susceptibility to PM. Burchenal and co-workers (2) reported a strain of *S. faecium* resistant to 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (DDMP) which was cross-resistant to MTX. Although bacteria resistant to MTX or aminopterin have been studied extensively (9), few of these strains have been examined for their resistance patterns to the typical antimetabolic or antibacterial antifolate compounds. An MTX-resistant strain of *Escherichia coli* has been reported to be cross-resistant to PM (F. M.

FIG. 3. Development of patterns of resistance when *Pedococcus cerevisiae* was exposed to increasing concentrations of chlorguanide triazine (CGT), pyrimethamine (PM), or trimethoprim (TMP).

Schabel, G. P. Wheeler, and H. E. Skipper, Proc. Amer. Ass. Cancer Res. 2:43-44, 1955), and strains of *S. faecium* and *P. cerevisiae* resistant to MTX showed cross-resistance to DDMP (2, 10).

With few exceptions, the previous studies have been concerned only with the organisms at the most resistant point, permitting no insight into the nature or complexity of the development of resistance patterns. Therefore, in the present studies we examined not only the final most resistant strains but also representative strains isolated and preserved at various stages during the elaboration of resistance. The results of these studies warrant certain conclusions.

(i) Development of resistance to antimalarial antifolate agents in these bacteria appears to comprise a number of discrete steps. These occur rather unpredictably in time but lead to the formation of a series of stable mutant strains with unchanging characteristics. These variants of intermediate resistance as well as the most highly nonsusceptible forms have exhibited consistently the same patterns of resistance, cross-resistance, and collateral susceptibility observed when they were first isolated 3 to 5 years ago.

(ii) The mutants in most cases were cross-resistant to all compounds investigated, but the quantitative relationships were unpredictable. Thus, the degree of resistance to one compound was not an accurate indication of the degree of resistance to another of the drugs. Similarly, Greenberg and Bond (7) observed a "lack of proportional increase in resistance" in tests with PM-resistant strains of *Plasmodium gallinaceum* when tested against other antifolates. Thompson and Bayles (19) reported two strains of *P. berghei* which had approximately the same resistance to PM but which differed substantially in resistance to CGT.

(iii) Resistant strains were obtained more easily upon exposure of these bacteria to CGT than upon exposure to either PM or TMP. It is interesting that strains of *S. faecium* and *P. cerevisiae* resistant to CGT were cross-resistant to PM and TMP, whereas strains of *L. casei* resistant to CGT were collaterally susceptible to the other two compounds. Confirmation of the collateral susceptibility in *L. casei* has been reported (18).

(iv) Resistance to these antifolate compounds which are active antimalarials was accompanied by only low-grade cross-resistance to MTX, an active antileukemic agent. Strains of *L. casei* resistant to CGT and collaterally susceptible to PM and TMP were also collaterally susceptible to MTX.

The studies reported here substantiate conclusions of several workers that resistance to the

antifolates is very complex and probably can be attributed to a variety of mechanisms (8, 9).

The biochemical bases for the development of these resistant strains are under investigation. Studies on the dihydrofolate reductases and thymidylate synthetases of the most highly resistant strains of *S. faecium* have been reported (6). Increases over the parent susceptible strain in dihydrofolate reductase activity ranged from 2- to 9-fold and in thymidylate synthetase activity from 10- to 12-fold. However, for the *S. faecium* TMP_r-G resistant strain, no change in the level of either enzyme activity could be detected. The dihydrofolate reductase activity in the PM_r-F resistant strain was increased and the enzyme exhibited a lower K_m and a pH maximum at 5, which was one whole pH unit lower than the maximum (pH 6) for the enzymes from the parent strain or from the other resistant strains.

Some transport studies have been completed (B. W. Siegel, C. S. Genthner, and C. C. Smith, Fed. Proc., p. 807, 1970). The most highly resistant strains of *S. faecium* and *L. casei* showed no differences from the susceptible strains in transport of radiolabeled CGT, PM, or TMP. Strains of *S. faecium* highly resistant to CGT and PM showed increased uptake of folic acid and MTX when compared with the strains of lower resistance. No differences in folic acid or MTX uptake were detected between the susceptible and the most resistant strains of *S. faecium* resistant to TMP, or *L. casei* resistant to CGT or PM.

The combined effects of all of these changes do not appear to account for the total drug resistance exhibited by these organisms. Further studies on the growth requirements and on biochemical differences in these strains are in progress. It is our hope that by analyzing the biochemical changes as one moves from one intermediate strain to the next and finally to the most resistant strains we will be able to account for the total fold increase in resistance. Study of the changes in the *P. cerevisiae* strain resistant to CGT may make it possible to explain why this organism readily develops resistance to CGT but shows no significant change in susceptibility to PM or TMP after prolonged exposure to these latter agents.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of John H. Roszmann, Roseanne Durig, and Larry M. Ayres.

This investigation was supported by Army contracts DADA17-67-C-7065 and DADA17-71-C-1011.

LITERATURE CITED

1. Bishop, A. 1966. A study of resistance to cyloguanil in *Plasmodium gallinaceum* in chicks. Parasitology 56:335-345.
2. Burchenal, J. H., G. B. Waring, and D. J. Hutchison. 1953.

- Development of resistance to 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (SK 5265) by *Streptococcus faecalis*. Proc. Soc. Exp. Biol. Med. 82:536-538.
3. Deibel, R. H., D. E. Lake, and C. F. Niven, Jr. 1963. Physiology of the enterococci as related to their taxonomy. J. Bacteriol. 86:1275-1282.
 4. Flynn, L. M., V. B. Williams, B. L. O'Dell, and A. G. Hogan. 1951. Medium for assay of vitamins with lactic acid bacteria. Anal. Chem. 23:180-185.
 5. Foley, G. E., and P. L. Watson. 1953. Chemical and biological studies on 1,2-dihydro-s-triazines. VI. Development of resistance by *Streptococcus faecalis* No. 8043. Proc. Soc. Exp. Biol. Med. 83:742-748.
 6. Freisheim, J. H., C. C. Smith, and P. M. Guzy. 1972. Dihydrofolate reductase and thymidylate synthetase in strains of *Streptococcus faecium* resistant to pyrimethamine, chlorguanide triazine, trimethoprim and amethopterin. Arch. Biochem. Biophys. 148:1-9.
 7. Greenberg, J., and H. W. Bond. 1956. Further studies on cross-resistance between pyrimethamine and related compounds. Amer. J. Trop. Med. Hyg. 5:14-18.
 8. Hitchings, G. H., and J. J. Burchall. 1965. Inhibition of folate biosynthesis and function as a basis for chemotherapy. Advan. Enzymol. 27:417-468.
 9. Hutchison, D. J. 1963. Cross resistance and collateral sensitivity studies in cancer chemotherapy. Advan. Cancer Res. 7:235-350.
 10. Hutchison, D. J., and J. H. Burchenal. 1952. Development of resistance to 4-amino-N¹⁰-methyl-pteroylglutamic acid (amethopterin) by *Leuconostoc citrovorum*. Proc. Soc. Exp. Biol. Med. 80:516-518.
 11. Johns, D. G., and J. R. Bertino. 1965. Folates and megaloblastic anemia: a review. Clin. Pharmacol. Ther. 6:372-392.
 12. Nichol, C. A. 1968. Studies on dihydrofolate reductase related to the drug sensitivity of microbial and neoplastic cells. Advan. Enzyme Regul. 6:305-322.
 13. Peters, W. 1969. Drug resistance in malaria—a perspective. Trans. Roy. Soc. Trop. Med. Hyg. 63:25-44.
 14. Rollo, I. M. 1955. The mode of action of sulphonamides, proguanil and pyrimethamine on *Plasmodium gallinaceum*. Brit. J. Pharmacol. 10:208-214.
 15. Schmidt, L. H. 1969. Chemotherapy of the drug-resistant malaras. Annu. Rev. Microbiol. 23:427-454.
 16. Schmidt, L. H., J. Harrison, R. Ellison, and P. Worcester. 1969. Activities of trimethoprim against infections with pyrimethamine susceptible and resistant strains of *Plasmodium cynomolgi*. Proc. Soc. Exp. Biol. Med. 131:294-297.
 17. Singer, S., G. B. Elion, and G. H. Hitchings. 1966. Resistance to inhibitors of dihydrofolate reductase in strains of *Lactobacillus casei* and *Proteus vulgaris*. J. Gen. Microbiol. 42:185-196.
 18. Smith, C. C., and C. S. Genther. 1971. Confirmation of collateral sensitivity to pyrimethamine in *Lactobacillus casei*, ATCC 7469 resistant to chlorguanide triazine. Ann. N.Y. Acad. Sci. 186:185-186.
 19. Thompson, P. E., and A. Bayles. 1968. Reciprocal cross resistance between cycloguanil hydrochloride and pyrimethamine in *Plasmodium berghei* infections in mice. J. Parasitol. 54:588-593.
 20. World Health Organization. 1967. Chemotherapy of malaria. World Health Organ. Tech. Rep. Ser. No. 375.