R-FACTOR TRIMETHOPRIM RESISTANCE MECHANISM: AN INSUSCEPTIBLE TARGET SITE

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SUMMARY R-factor R388 increases the resistance of Escherichia coli to trimethoprim by 10,000 fold, and mediates the synthesis of an additional dihydrofolate reductase that is less susceptible to trimethoprim by a similar order of magnitude. The dihydrofolate reductase conferred by the R-factor was of a larger molecular weight than the wild-type enzyme and exhibited a different pattern of response to trimethoprim inhibition. This is thought to be the first example of an R-factor conferring an altered target site mechanism of resistance to a chemotherapeutic agent.

Trimethoprim, a selective inhibitor of bacterial dihydrofolate reductase (EC.1.5.1.3.) has been used clinically in combination with sulfonamides for about five years. Since its introduction, R-factors conferring high-level resistance to trimethoprim have been detected in clinical strains of bacteria (1,2). Previous knowledge concerning the mechanisms by which R-factors mediate resistance to other drugs suggests that drug inactivation or impermeability would be the most likely candidates when considering a new plasmid resistance mechanism (3). However, it is known that E.coli can mutate to aminopterin- or trimethoprim-resistance either by increasing production of normal dihydrofolate reductase (4,5) or by synthesizing a drug-resistant form of the enzyme (6). In addition, an amethopterin-resistant

mutant of Streptococcus faecalis was found to contain a drug-resistant dihydrofolate reductase as well as its normal complement of wild-type enzyme (7). It is hence possible that trimethoprim-resistance could be conferred by an R-factor by means of an altered dihydrofolate reductase.

MATERIALS AND METHODS The prototropic E.coli strain 114 was used as it is drug-sensitive (8). R-factor R388 was kindly supplied by Dr. N. Datta and transferred into strain 114. NADPH was obtained from PL-Biochemicals Ltd., Milwaukee, U.S.A., dihydrofolate from Sigma Chemical Co., London, U.K., Sephadex G-75 from Pharmacia, Uppsala, Sweden. Trimethoprim was kindly given by the Wellcome Research Laboratories.

Dihydrofolate reductase activity was assayed by the method of Osborn and Huennekens (9). Reactions were performed at 30°C in an Optika CF4R spectrophotometer. The assay mixture was 40 mM sodium phosphate buffer pH 7.5, 0.08 mM NADPH, 10 mM β-mercaptoethanol, enzyme, trimethoprim where necessary and distilled water to 2.3 ml. After four minutes for temperature equilibration, the reaction was started by adding dihydrofolate to 0.08 mM and the absorbancy at 340 nm followed. One unit of enzyme activity was the amount causing a decrease in optical density of 0.01 per minute under the above conditions (9). Protein concentrations were estimated by the method of Waddell (10).

The enzyme was prepared from 18 liter batches of exponential phase bacteria grown aerobically in minimal medium (11) with 0.28% glucose as a carbon source. All subsequent operations were carried out at 0-4°C. The cells were harvested by centrifugation and washed twice with growth medium lacking glucose. The pellet was resuspended in 10 mM β-mercaptoethanol and 1 mM EDTA in 20 mM sodium phosphate buffer pH 7.4, then treated in a M.S.E. ultrasonic disintegrator for 3 x 1 minute. The disrupted suspension was centrifuged at 18,000 r.p.m. for one hour and the pellet discarded. Nucleic acids were precipitated by the addition of 0.1 volume 10% streptomycin sulfate. After centrifugation ammonium sulfate was added to 50% saturation. The precipitate was removed by centrifugation at 10,000 r.p.m. for 20 minutes and discarded. Ammonium sulfate was added to give 80% saturation. After centrifugation the supernatant was discarded and the pellet containing virtually all the dihydrofolate reductase activity was dissolved in 10 mM β-mercaptoethanol and 1 mM EDTA in 50 mM sodium phosphate buffer pH 7.4 (buffer A). After overnight dialysis against 100 volumes of buffer A the preparation containing the dihydrofolate reductase activity was applied to a column

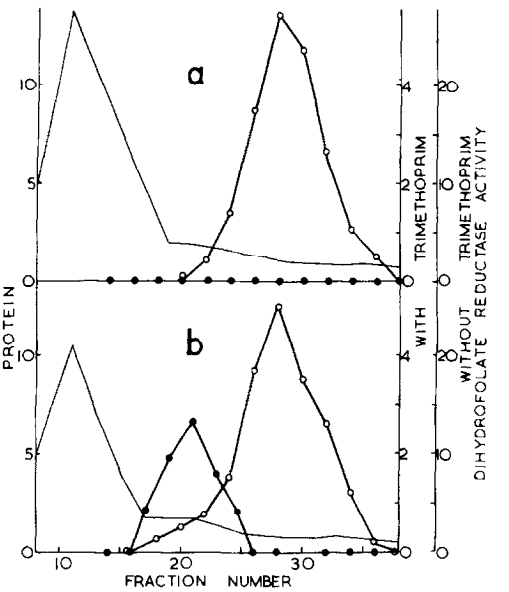

FIGURE 1. Elution of dihydrofolate reductase activities of E.coli 114 (a) and E.coli 114 (R388) (b). ——— Protein in mg/ml, O——O total dihydrofolate reductase activity and ●——● dihydrofolate reductase activity in the presence of 4 x 10^{-6}M trimethoprim. Enzyme activity refers to dihydrofolate reductase level in units/ml.

(4 x 60 cm) of Sephadex G-75 and eluted with buffer A. The minimum inhibitory concentrations for trimethoprim were determined as previously described (8).

RESULTS The minimum inhibitory concentration (M.I.C.) of trimethoprim for E.coli 114 was found to be 0.2 μg/ml on Davis-Mingioli medium. However, when R388 was transferred into E.coli 114 the M.I.C. was 3000 μg/ml thus producing an increase of resistance of over 10,000 fold.

R388 did not significantly affect the level of dihydrofolate reductase activity of E.coli 114 even when strain 114 (R388) was grown in medium containing trimethoprim (5 μg/ml). Thus there

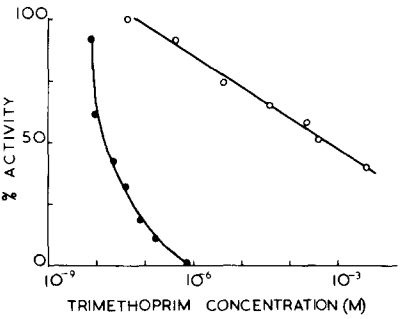

FIGURE 2. Effect of trimethoprim on the dihydrofolate reductases obtained after gel filtration.
●——● E.coli 114 [fractions 25-35]
○——○ E.coli 114(R388)[fractions 18-20]

was no evidence of induction of dihydrofolate reductase nor did the level of enzyme appear to contribute to trimethoprim-resistance, as found in some mutant bacteria (4).

The dihydrofolate reductase of E.coli 114, and of E.coli 114 (R388) was fractionally precipitated with ammonium sulfate. The result with either strain was that 50% saturation precipitated NADPH oxidase activity without appearing to remove dihydrofolate reductase. It is technically difficult to estimate low levels of dihydrofolate reductase in the presence of NADPH oxidase. The 50-80% ammonium sulfate precipitate from either strain contained virtually all its dihydrofolate reductase activity. After dialysis these were subjected to gel filtration on Sephadex G-75 and a single peak of activity was found in strain 114 (Fig. 1a). Portions of fractions 25-35 were pooled and precipitated with ammonium sulfate at 80% saturation. The pellet was dissolved in buffer A and its sensitivity to trimethoprim tested. It was

found that 1.8 x 10^{-8}M trimethoprim reduced the activity by 50% (Fig. 2).

The 50-80% ammonium sulfate precipitate from strain 114(R388) was similarly subjected to gel filtration on Sephadex G-75. Fractions were assayed for dihydrofolate reductase and a peak of enzyme activity, similar to that obtained in strain 114, was found in strain 114 (R388) (Fig. 1). When the assays were repeated in the presence of 4 x 10^{-6}M trimethoprim (a concentration sufficient to abolish the activity of the wild-type enzyme, see Fig. 1a) a trimethoprim-insensitive enzyme was revealed (Fig. 1b). The total activity in the trimethoprim-insensitive peak was lower than that for the wild-type enzyme. Fractions 18-20 containing the trimethoprim-insensitive activity, but not wild-type enzyme, were pooled and precipitated with ammonium sulfate at 80% saturation. The concentration of trimethoprim required for 50% inhibition was found to be 4 x 10^{-4}M which is about 20,000 times more resistant than the enzyme from strain 114. It can be seen (Fig. 2) that the inhibition of the trimethoprim-insensitive enzyme from strain 114 (R388) was linear with respect to logarithmic trimethoprim concentration, while that of the wild-type enzyme was not; suggesting the enzymes differ in their binding characteristics.

The molecular weights of the two dihydrofolate reductase enzymes were estimated by gel filtration on Sephadex G-75 using chymotrypsinogen, ovalbumin and cytochrome C as markers (12). The molecular weight of the wild-type enzyme was found to be

21,000 in either strain, which agrees with the published value (13). The trimethoprim-insensitive enzyme of strain 114 (R388) had a molecular weight of 35,000.

DISCUSSION We show that R388 increased the resistance of E.coli to trimethoprim by about 10,000 fold and mediated the synthesis of an additional dihydrofolate reductase which was about 20,000 times less susceptible to trimethoprim inhibition. We suggest this enzyme is responsible for the trimethoprim-resistance conferred by R388. We have performed additional experiments and cannot detect an R-factor mediated enzyme that inactivates the drug, nor do we find the strains differ in their uptake of trimethoprim. Hence an altered target site seems to be the sole mechanism of resistance to trimethoprim mediated by R388. This mechanism may be the first example of an altered target site being the mode of R-factor mediated resistance to a chemotherapeutic agent.

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