Effect of trimethoprim on folate-dependent DNA synthesis in human bone marrow

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SYNOPSIS The effect of trimethoprim in various concentrations was tested on the folate-dependent deoxyuridylicate methylation reaction in human bone marrow cultures. A slight effect was demonstrated at a concentration similar to that achieved in the plasma in patients taking the conventional therapeutic dose of trimethoprim. When a tenfold greater concentration was used, the effect became more pronounced. These findings are in agreement with reports of some mild haematological changes associated with the use of trimethoprim and offer an explanation for such changes.

Septrin (Burroughs Wellcome & Co), a new, widely used antibacterial drug, is a combination of trimethoprim and sulphamethoxazole. Trimethoprim is seldom if ever used alone. It acts as a bacteriostatic agent by competitively inhibiting bacterial folate reductase activity (Hitchings, Falco, Vanderwerff, Russell, and Elion, 1952). In order to produce a similar degree of inhibition of the human enzyme, a concentration of the drug 50000 times greater is required (Hitchings, 1969). Because of this, it might be anticipated that trimethoprim in the recommended therapeutic dose of 320 mg daily would not have a measurable effect on folate metabolism in humans. However, interference with folate metabolism has been reported in some patients receiving trimethoprim. Kahn, Fein, and Brodsky (1968) noted a fall in haemoglobin concentration, platelet count, and serum folate, together with excessive formimino-glutamicaciduria and bone marrow changes, in a high percentage of patients receiving 1 g trimethoprim daily. Small but significant changes in haemoglobin concentration, neutrophil count, and serum folate concentration were also observed in patients taking 500 mg daily (Jenkins, Hughes, and Hall, 1970). A daily dose of 200 mg did not produce significant changes in any of these haematological parameters (Whitman, 1969). In order to obtain further information on the effect of trimethoprim on folate metabolism, in vitro studies were undertaken on the effect of trimethoprim on folate-dependent DNA synthesis via the methylation of deoxyuridylate to thymidylate. The bone marrow culture system originally described by Killman (1964) was used. In this system, unlabelled deoxyuridine is added to bone marrow cultures. In the presence of adequate amounts of tetrahydrofolic acid, the deoxyuridine is methylated to form thymidine. The resulting increase in the thymidylate pool blocks the incorporation of subsequently added tritiated thymidine into DNA (Fig. 1). Such a system has been used to assess the

![Diagram](http://jcp.bmj.com/)

**Fig. 1 Synthesis of deoxyribonucleic acid (DNA)-thymine and site of blockade by trimethoprim. (After Waxman, Corcino, and Herbert, 1970).**

folate-dependent methylation of deoxyuridylicate in a number of studies (Metz, Kelly, Swett, Waxman, and Herbert, 1968; Waxman, Metz, and Herbert, 1969; Waxman and Herbert, 1969). Since the reaction

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involves hydrogen transfer as well as methylation, it is dependent on continued regeneration of tetrahydrofolic acid by the enzyme dihydrofolate reductase and is extremely sensitive to the action of a dihydrofolate reductase inhibitor such as methotrexate (Metz et al., 1968). For this reason, the system was chosen to detect any possible effect of trimethoprim, and the reversibility of such an effect by tetrahydrofolic acid.

Material and Methods

The antibacterial potency of the trimethoprim used was checked by testing its bacteriostatic effect on the Oxford strain of Staphylococcus. As trimethoprim is poorly soluble in water, it was dissolved in ethanol. Solutions of trimethoprim from $10^{-5}$ to $1 \text{ mM}$ were prepared in ethanol concentrations which ranged from 0.001% to 4%.

Samples of bone marrow were obtained from haematologically normal patients either by aspiration from the posterior iliac crest or from ribs removed at thoracotomy. Marrow cultures were set up in autologous plasma as described previously (Metz et al., 1968). Aliquots (0-6 ml) of marrow cells were pipetted into tubes, and the various non-radioactive and radioactive components added in 0-1 ml volumes, according to the individual experiments. The amount of deoxyuridine added was $1 \mu\text{M}$. The final volume in each tube was 1 ml. Trimethoprim solutions were added in 0-1 ml volumes, so that the final concentration in the system was one-tenth the concentration of the solution added. (All subsequent reference to concentrations of trimethoprim refer to final concentration in the test system.) The cultures were incubated with $1 \mu\text{Ci}$ aliquots of thymidine-$^3\text{H}$-methyl tritiated thymidine (specific activity 18-28 Ci/mMole) for three hours at 37°C. Following incubation, the cells were washed twice in 2 ml volumes of 0-85% buffered saline. The cells were then reconstituted in a final volume of 1 ml. After mixing 0-1 ml volumes of the cell suspension were 'spotted' on filter paper discs, and the discs were allowed to dry. After drying, the discs were placed in a large volume (200 ml) of 10% trichloracetic acid allowed to stand at 4°C for 20 minutes. The discs were then removed and swirled around in a further 200 ml of 10% trichloracetic acid, and then washed twice in 200 ml volumes of 3% perchloric acid, followed by 74% ethanol, and finally ether. After they were dried, the radioactivity on the discs was determined using a Packard tricarb liquid scintillation counter (model 3003). All samples were counted to an accuracy of 1%. Quenching was monitored using a built-in external standard.

Since trimethoprim used was dissolved in varying concentrations of ethanol, preliminary experiments were carried out to determine the effect, if any, of similar concentrations of ethanol on the marrow cultures. In two normal marrows, concentrations of alcohol from 0.0001% to 4% failed to affect the incorporation of tritiated thymidine into DNA either with or without previously added deoxyuridine. These results agree with the findings of Corcino, Waxman, Rubin, and Herbert (1970) who reported that ethanol does not affect the incorporation of thymidine into DNA in bone marrow cultures. Nevertheless, control cultures containing the appropriate amounts of ethanol were included in all experiments.

Results

In nine normal marrows, the addition of deoxyuridine resulted in blocking the incorporation of tritiated thymidine to 11-9% or less (range 2-8-11-9%) of the uptake in the control tube without deoxyuridine. This figure is comparable to previous experience with this method (Metz et al., 1968).

When the effect of inhibitors was studied, these were added to the marrow cultures 30 min before the addition of the deoxyuridine. Final concentrations of trimethoprim ranging from $10^{-4}$ to $10^{-8} \text{ mM}$ failed to diminish the blocking effect of deoxyuridine (Fig. 2). A $10^{-4} \text{ mM}$ concentration of trimethoprim consistently produced slight inhibition of the deoxyuridine blocking effect, and $10^{-1} \text{ mM}$ a more marked effect (Fig. 2). This effect of trimethoprim was not the result of direct inhibition of tritiated thymidine incorporation into DNA, as no effect could be demonstrated in the absence of added deoxyuridine.

When 5-formyl tetrahydrofolic acid was added to a final concentration of $10^{-1} \text{ mM}$, the inhibition by trimethoprim $10^{-1} \text{ mM}$ of the blocking effect of deoxyuridine was reversed completely, whereas the addition of an equimolar amount of folic acid (PGA) had no effect (Fig. 3).

The effects of methotrexate and trimethoprim on deoxyuridylate methylation were compared in parallel experiments. Using equimolar concentrations of the two drugs, ranging from $10^{-4} \text{ mM}$ to $10^{-1} \text{ mM}$, it was found that $10^{-8} \text{ mM}$ methotrexate and $10^{-1} \text{ mM}$ trimethoprim produced inhibition of the blocking effect of deoxyuridine of a similar order (Fig. 4), so that 100000 times more trimethoprim than methotrexate is required to produce the same degree of inhibition of human dihydrofolate reductase.

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1Trimethoprim was obtained as the lactate salt from Burroughs-Wellcome by courtesy of Dr S. R. M. Bushby.
Fig. 2 The effect of various concentrations of trimethoprim on deoxyuridine suppression of $^3$H-thymidine incorporation into DNA in human bone marrow cultures. Each pair of columns within a single concentration of TMP represents an individual experiment on a different marrow sample. $10^{-4}$ mM is approximately the concentration found in the plasma of patients receiving conventional therapeutic doses of the drug. $dU =$ deoxyuridine; $TMP =$ trimethoprim

Fig. 3 The effect of reduced (5-HCO THFA) and non-reduced (PGA) folate on the DHFR inhibitory action of trimethoprim. The trimethoprim effect is reversed by reduced folate only.

dU = deoxyuridine
$TMP =$ trimethoprim
5-HCO-THFA = 5-formyl tetrahydrofolate
$PGA =$ pteroylglutamic acid

Discussion

The exact mechanism whereby folate deficiency and folate antagonists bring about changes in haemopoietic cells is unknown. Current thought favours the concept of 'thymine-less' death of cells, resulting from failure to methylate deoxyuridylate to thymidylate (Pastore and Friedkin, 1962). The dihydrofolate-reductase dependent methylation of deoxyuridylate is a key coenzymic function of folate, and the results of the present study indicate that trimethoprim in sufficiently high concentration can inhibit this reaction in human bone marrow culture. The observation that this effect is reversed by reduced folate (5-formyl tetrahydrofolic acid) and not by the non-reduced PGA provides substantive evidence...
that it is due to inhibition of dihydrofolate reductase.

The conventional therapeutic dose of trimethoprim is 160 mg twice daily. This dose produces midpoint plasma concentrations of the drug ranging from 0.8 to 2.75 µg/ml (Sharpestone, 1969). Steady state levels average 2 µg/ml (Miller and Salter, 1971). A concentration of the same order of magnitude (10^{-2} mM equivalent to 2.79 µg/ml) in the marrow culture system produced only a minimal effect on deoxyuridylate methylation. These experimental results are in good agreement with clinical experience with the drug, for a dose of 250 mg twice daily, which is some one and a half times greater than the conventional therapeutic dose, produces only minor haematological changes (Jenkins et al, 1970). In patients receiving 250 mg of trimethoprim four times daily, a dose three times the conventional therapeutic dose, the mild megaloblastic changes observed in a high proportion of patients and the fall in haemoglobin level (Kahn et al, 1968) are in accord with our findings in vitro using greater concentrations of the drug.

It is possible to conclude that the reported effects of large doses of trimethoprim on the haemopoietic system are probably the result of interference with the methylation of deoxyuridine arising from inhibition of dihydrofolate reductase. The amount of inhibition produced by conventional therapeutic doses is unlikely to be of clinical significance. However, the therapeutic margin of safety is relatively narrow, and for this reason, the likelihood of haematological side effects is greater both in patients with suboptimal folate nutrition and in patients with impaired renal function.

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References


Addendum

Since submitting the paper for publication, an abstract reporting similar findings has appeared (Mechanism of trimethoprim—induced deranged folic acid metabolism in human bone marrow. S. Waxman, Clin. Res., 19, 434, 1971). This author showed inhibition of dihydrofolate reductase by TMP in human bone marrow cultures, and recommends that the drug be used with care in patients with low folate stores.