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THE *IN VITRO* EFFECT OF TRIMETHOPRIM ON BLASTOGENIC TRANSFORMATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Abstract—Trimethoprim (TP) at concentrations of 1×10^{-6} to 1×10^{-3} M inhibits the incorporation of thymidine, uridine, formate, and leucine by human lymphocytes undergoing blastogenic transformation induced by conconavalin A. TP does not alter cell viability, as measured by trypan blue exclusion, or cell numbers. An effect of TP on dihydrofolate reductase, such as is seen with methotrexate, is insufficient to explain the inhibitory effects of TP since an *in vivo* estimation of dihydrofolate reductase activity, utilizing deoxyuridine incorporation into deoxyribonucleic acid, is not inhibited. Also the observed inhibition of precursor incorporation is not fully reversed by either folate or folinate. TP did not alter the percentage of cells transforming but reduced the number of autoradiographically-labelled lymphoblasts. Immunosuppression by TP *in vivo* may be associated with these findings.

Trimethoprim (2,4-diamino-5,3,4,5-trimethoxybenzoyl pyrimidine; TP) has a potent immunosuppressive effect in preventing the rejection of skin allografts in mice [1] and may be immunosuppressant in man [2]. We have investigated the effect of this substance on the transformation produced in normal human peripheral lymphocytes by the plant lectin, concanavalin A (Con A). This mitogen induces lymphocytes in culture to increase their synthesis of RNA, DNA and protein with associated cellular enlargement and mitosis, i.e. blastic transformation. This process is believed to reflect some of the early steps in an immune response. The population of cells responding in this fashion, however, is larger and probably different from that responding in a similar way to a specific antigenic stimulus [3]. A more specific type of response is evoked by allogeneic lymphocytes during the co-cultivation of lymphocytes from genetically unrelated donors (mixed lymphocyte cultures) and the effect of TP has also been investigated in such cultures.

MATERIALS AND METHODS

Trimethoprim was a gift of Dr. J. J. Burchall (Burroughs Wellcome Co., Research Triangle Park, NC). Concanavalin A was a lyophilised, three-times crystallised preparation from Miles–Yeda (Kankakee, IL). 2-deoxyuridine and folic acid were obgained from Sigma Chemical Company (St. Louis, MO). Methyl-α-D-mannopyranoside was from Calbiochem (LaJolla, CA); 0.4% Trypan Blue in normal saline from Gibco (Grand Island, NY) and methotrexate sodium from Lederle (Pearl River, NY). Eagle's Minimal Essential Medium, Hank's balanced salt solution and fetal calf serum were purchased from Microbiological Associates (Bethesda, MD). Folate-free Minimal Essential Medium was prepared and sterilised in our laboratory. PCS is a product of Amersham–Searle (Arlington Heights, IL).

The following radioisotopes were obtained from the New England Nuclear Corporation (Boston, MA) and were diluted in Hank's solution to allow the addition of 1 μCi to each culture in a volume of 20 μL: thymidine [^{3}H methyl] (sp. act. 6.7 Ci/mM), [5,6-^{3}H]uridine—(sp. act. 45 Ci/mM) and [1-^{14}C]-L-leucine (sp. act. 25 mCi/mM). [3′, 5′-^{3}H]folic acid (sp. act. 22 Ci/mM) from Schwarz–Mann (Orangeberg, NY) was diluted in 0.5% sodium bicarbonate to allow addition of 1 μCi/ml culture. The purity of this compound was checked by paper chromatography before use. [^{14}C]Sodium formate (sp. act. 58 mCi/mM) was purchased from Amersham–Searle (Arlington Heights, IL).

Isolation, culture and chemical extraction of human lymphocytes. Heparinised peripheral blood from normal subjects was sedimented by gravity in sterile tubes at 37°. The leucocyte-rich plasma was periodically aspirated and the cells washed twice in Eagle's Minimal Essential Medium (MEM). The lymphocyte concentration was determined by counting in a haemocytometer using 0.1% crystal violet in 1% acetic acid and the final volume of the suspension was adjusted to give 10^6 lymphocytes/ml medium which was enriched with 10% v/v fetal calf serum. One milliliter cultures were set up in 12 × 75 mm polyethylene culture tubes (Falcon No. 2051, Falcon Plastics, Oxnard, CA) and were usually stimulated with 40 μg Con A in Hank's Balanced Salt Solution. Drugs or diluents were added at the times indicated. TP was added after solution in ethanol. Cultures were set up in triplicate and incubated at 37° in a water-saturated atmosphere of 5% carbon dioxide in air, usually for 72 hr. Isotopically labelled precursors were added at

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the times indicated and incubation was terminated by the addition of 3 ml ice-cold Hank's solution. The cells were harvested by centrifugation and washed a second time in this medium and then successively in two, 3 ml aliquots of ice-cold 5% trichloracetic acid (TCA) and finally in 3 ml methanol. The residue was dissolved in 0.5 ml 1 M Hyamine-10-X in methanol at 70° for 15 min and transferred to counting vials with 3 ml ethanol. A scintillation mixture (3 g 2,5-diphenyloxazole (PPO), 100 mg 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP), 300 ml ethanol and 700 ml toluene) was added and the vials counted in a Beckman liquid scintillation system with an efficiency of approximately 20% for ^3H and 80% for ^{14}C. Where appropriate, disintegrations per min were calculated using an external standard channels ratio method.

Two-way mixed lymphocyte cultures were set up using lymphocytes prepared as above from two unrelated donors, 5×10^5 lymphocytes from each donor being mixed to give 1 ml culture. These were incubated for 6 days, [^{3}H]thymidine being present during the final 3 hr of culture. Preparation for counting was as described above.

Folate and folinate studies. The effect of TP on folic acid uptake during transformation was studied using 10^6 lymphocytes/ml culture in folate-free MEM. After 67 hr incubation 1 μCi [^{3}H]folic acid was added and at 72 hr 3 ml ice-cold Hank's solution added. The cells were washed twice more in ice-cold Hank's solution, the supernatant aspirated and 1 ml water added to lyse the cells which were held at 70° for 20 min. 5 ml PCS (Amersham–Searle) was added to the lysate in a counting vial and the mixture counted in the liquid scintillation spectrometer.

In experiments in which the folate or folinate concentration of the medium was varied the cells, after initial sedimentation, were washed twice in medium containing the desired folate or folinate concentration and cultures set up and processed as described previously.

Deoxyuridine suppression test. The deoxyuridine suppression test was carried out by the method of Metz *et al.* [4]. 2-deoxyuridine was added to 3-day stimulated lymphocyte cultures to give final concentrations of 4–4 $\times 10^{-3}$ μM/ml and an appropriate concentration of TP was added. One hr later 1 μCi [^{3}H]thymidine was added and after a further 3 hr the cells were harvested and processed as above.

Cell counting and autoradiography. To estimate cell numbers, clumping was first reduced by adding methyl-α-D-mannopyranoside in Hank's solution to give a final concentration of 50 mM/ml culture and incubation continued for a further hr. After gentle agitation, cells were stained with 0.1% crystal violet in 1% acetic acid and counted in a haemocytometer. Cell viability was estimated by counting the percentage of cells excluding Trypan Blue [5]. Autoradiography was carried out on preparations made from 72-hr cultures labelled for 3 hr with 1 μCi [^{3}H]thymidine, fixed in 3:1 (v/v) methanol–acetic acid, and exposed to Kodak Nuclear Track Emulsion Type NTB-2 for 7 days before development. The slides were then stained with Giemsa stain and examined under oil-immersion: at least 1250 cells were examined for blastic transformation.

Analysis. Data were analyzed by the Mann–Whitney U-test. The 0.05 level of probability was accepted as significant.

RESULTS

TP at concentrations ranging from 1×10^{-6} M produced a dose-dependent inhibition of the increase in [^{3}H]thymidine incorporation induced in lymphocytes by Con A (Fig. 1). In the presence of 5×10^{-5} M TP, thymidine incorporation was reduced to 71 ± 1.5% (S.E.M. $n = 8$) of control values, this being the concentration approximately attained in human tissues where concentration of TP occurs. At 1×10^{-3} M the incorporation of thymidine is almost completely suppressed. The effect of the ethanol, used as the solvent for TP, was trivial and the mean incorporation of thymidine into lymphocytes stimulated with Con A was 9% less in those cultures exposed to ethanol. In all experiments control cultures were set up containing ethanol at the same concentration as was used as the vehicle for TP.

When the incorporation of [^{3}H]thymidine into DNA was determined at different times over a period of 143 hr it was found that the inhibitory effect of TP was maintained over this time. Figure 2 shows that TP 1×10^{-4} M significantly suppresses thymidine incorporation at 43, 70, and 143 hr.

Lymphocytes stimulated into blastic transformation by a two-way lymphocyte reaction exhibited a similar dose-dependent inhibition of thymidine incorporation by TP (Fig. 3).

TP had a reduced inhibitory effect on [^{3}H]thymidine incorporation when its contact time with the cells was short. For example 1×10^{-4} M TP reduced thymidine incorporation to 42.2 ± 5% (S.E.M., $n = 12$) of control when present throughout incubation and when added one hour before [^{3}H]thymidine, i.e. contact time of 4 hr, the incorporation was 69.3 ± 3.7 (S.E.M., $n = 12$).

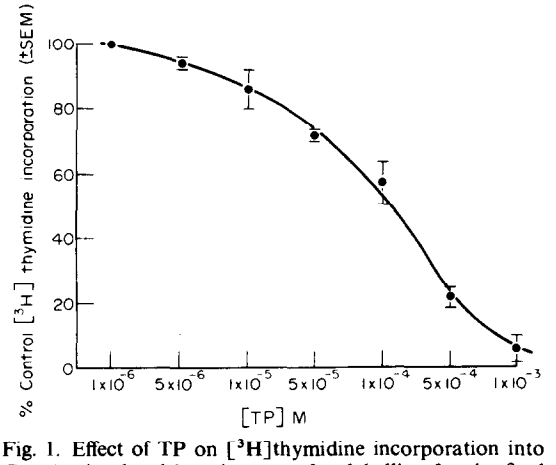

Fig. 1. Effect of TP on [^{3}H]thymidine incorporation into Con A-stimulated lymphocytes after labelling for the final 3 hr of 72 hr incubation at 37° (expressed as mean ± S.E.M. of the percentage incorporation relative to that of the untreated control). TP was added at the time of stimulation with 40 μg/ml Con A. Control cultures contained vehicle for TP in place of drug.

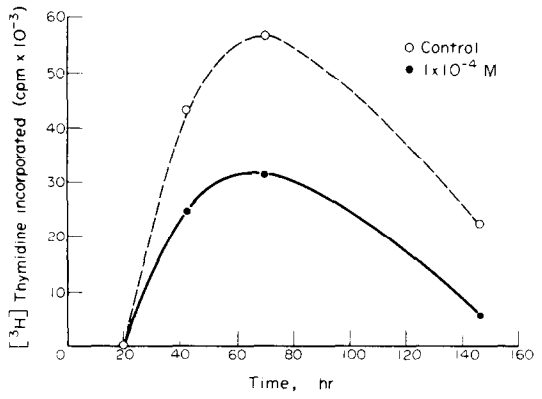

Fig. 2. Incorporation of [^{3}H]thymidine (as cpm. $\times 10^{-3}$) into Con A-stimulated lymphocytes after various incubation times with 1×10^{-4} M TP. TP was present throughout the culture period. The [^{3}H]thymidine was added for the final 3 hr of culture.

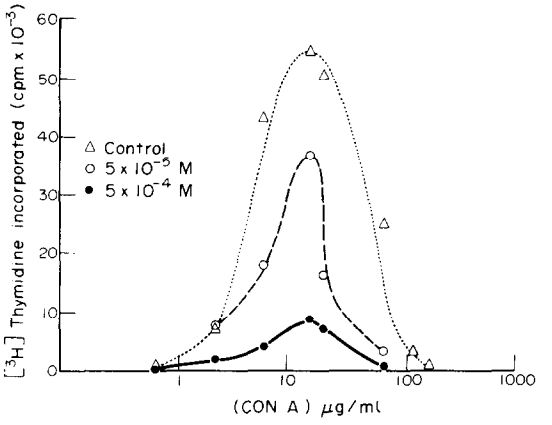

Fig. 4. Alteration of Con A concentration produces changes in [^{3}H]thymidine incorporation at 72 hr (as cpm $\times 10^{-3}$) in the absence (Δ) and presence of 5×10^{-4} M (●) and 5×10^{-5} M (○) TP. TP and Con A were present throughout culture, [^{3}H]thymidine was added for the final 3 hr of incubation.

Increasing the Con A concentration was unable to overcome the inhibitory effect of TP on thymidine incorporation as is shown in Fig. 4 which depicts the cellular response to varying concentrations of Con A plotted as a log dose–response curve. The inhibition of thymidine incorporation by higher Con A concentrations is a phenomenon attributed to toxic effects of the lectin [6]. In our experiments the mean viability of lymphocytes at 72 hr in the presence of 40 μg/ml Con A was 88 ± 2%. It is apparent from Fig. 4 that TP depressed the maximal response of the cells and in fact depressed the response at each level of Con A tested.

TP also inhibits the incorporation of [^{3}H]uridine (after 24 hr culture) and [^{14}C]formate (after 72 hr culture) into acid insoluble material in a dose-dependent manner as shown in Figs 5 and 6.

The overall rate of protein synthesis increases within a few hr of the initiation of lymphocyte transformation reaching a maximum of about 48 hr and then waning. Interestingly, TP showed much less inhibitory effect on the maximal 48-hr protein synthesis than at 72 hr (Fig. 7). This may reflect a secondary nature of the effect of TP protein synthesis.

Inhibition of [^{3}H]thymidine incorporation could result from a non-specific toxic effect on the cells affecting, for example, cell viability. Cellular viability, estimated by the trypan blue exclusion method [5], and cell counts, carried out in a haemocytometer, showed that drug treatment has no apparent effect on cell viability or numbers at 72 hr. To exclude the possibility of some more subtle interference with lymphocyte metabolism, which although it did not result in altered trypan blue permeability, was sufficient to permanently impair the cellular utilization of exogenous thymidine, a further experiment was carried out. Lymphocytes were pre-incubated with TMP for

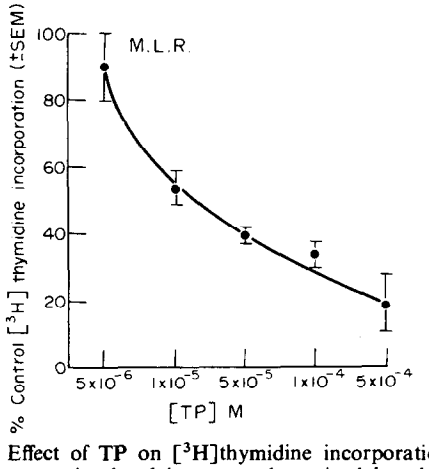

Fig. 3. Effect of TP on [^{3}H]thymidine incorporation of lymphocytes stimulated by a two-day mixed lymphocyte reaction. Lymphocytes from two unrelated donors incubated with and without TP for 6 days, [^{3}H]thymidine added for final 3 hr of incubation. Incorporation of [^{3}H]-thymidine expressed as mean ± S.E.M. of the percentage incorporation relative to control cultures containing vehicle for TP in place of drug.

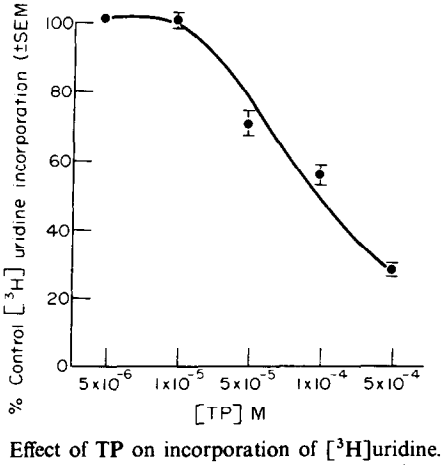

Fig. 5. Effect of TP on incorporation of [^{3}H]uridine. 10^6 cells/ml were incubated with Con A (40 μg/ml) and varying TP concentrations. After 21 hr [^{3}H]uridine (1 μCi) was added and the cells harvested at 24 hr. Incorporation of [^{3}H] into the acid-insoluble residue is expressed as mean ± S.E.M. of the percentage incorporation relative to that of the control cultures which contained the vehicle for TP in place of drug. Control incorporation was 25120 ± 950 cpm.

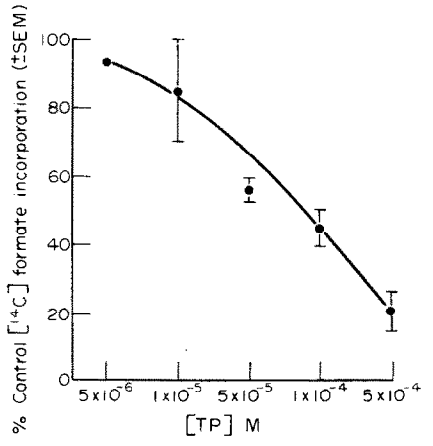

Fig. 6. Effect of TP on incorporation of [^{14}C]formate. 10^6 cells/ml were incubated with Con A (40 μg/ml) and varying TP concentrations. After 69 hr [^{14}C]formate (1 μCi) was added and the cells harvested at 72 hr. Incorporation of [^{14}C] into the acid-insoluble residue is expressed as mean ± S.E.M. of the percentage incorporation relative to that of the control cultures which contained the vehicle for TP in place of drug. Control incorporation was 9678 ± 652 cpm.

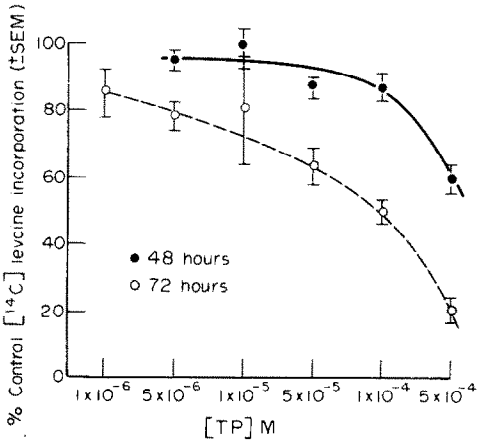

Fig. 7. Effect of TP on [^{14}C]leucine incorporation into Con A-stimulated lymphocytes after 48 (●) and 72 (○) hr incubation in presence of drug. Incorporation from a 3 hr pulse of [^{14}C]leucine (1 μCi) expressed as mean ± S.E.M. of percentage incorporation relative to controls. Controls contained vehicle for TP in place of drug.

24 hr at 37° before being washed twice with medium (with 2 hr at 37° between washes) to remove TP before their response to Con A at 48 hr was tested. Cells were also simply incubated in medium and washed as control. Cells exposed to 1×10^{-4} M TP before washing incorporated 10,966 ± 1075 cpm/10^6 cells ($n = 9$); control cultures which were washed and then stimulated, incorporated 10,385 ± 1457 cpm/10^6 cells ($n = 9$). A further group of control cultures which were pre-incubated without ethanol incorporated 9024 ± 973 cpm/10^6 cells ($n = 9$). These differences were not statistically significant. Therefore, no permanent damage seems to accrue from a 24 hr exposure to TP.

Effects of TP on the folate metabolism of transforming lymphocytes. One possible explanation of the effects of TP on the incorporation of isotopically labelled metabolic precursors might be an inhibition of lymphocyte dihydrofolate reductase.

An indirect estimate of the activity of dihydrofolate reductase *in vivo* may be obtained by the method of Metz *et al.* [4]. In this assay the synthesis of thymidylate from exogenous "cold" 2-deoxyuridine added to the transforming cells expands the intracellular pool of thymidylate. This reduces the specific activity of the exogenous [^{3}H]thymidine subsequently taken up during labelling so that the incorporation of tritium into DNA falls. Methotrexate (MTX) inhibits dihydrofolate reductase and so synthesis of thymidylate from deoxyuridine is reduced since this enzyme is required for rapid regeneration of tetrahydrofolate from dihydrofolate formed during the thymidylate synthetase reaction. The intracellular thymidylate pool is therefore depleted and exogenous [^{3}H]thymidine incorporation into DNA is increased. The effect is shown in Fig. 8. The effect of TP differed from that of methotrexate. These experiments suggest that TP does not inhibit the dihydrofolate reductase of transforming human lymphocytes.

Transforming lymphocytes show an increased folate uptake [7]. We studied this effect in folate-free medium at 72 hr and Table 1 shows the effect of TP on this process. We found a significant inhibition of nearly 20 per cent with 10^{-4} M MTX. TP, however, failed to diminish the folate uptake even at 5 times the concentration of MTX that did so.

An alternative assessment of dihydrofolate reductase activity would be by determination of the effects of addition of exogenous folate and folinate to cultures in the presence of TP. If TP acted by competitively inhibiting dihydrofolate reductase then increased exogenous folate should reduce the inhibition providing that TP does not alter folate permeability in lymphocytes. When folic acid (pteroylglutamic acid) or folinic acid (N^5-formyl tetrahydrofolic acid)

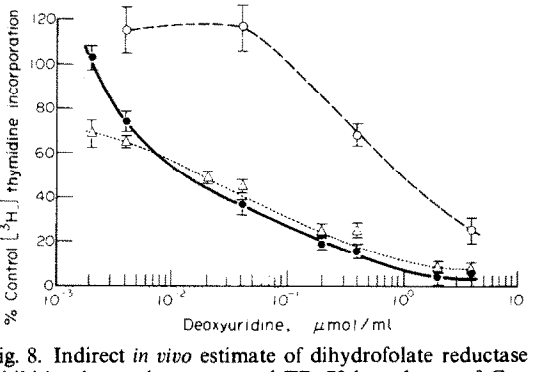

Fig. 8. Indirect *in vivo* estimate of dihydrofolate reductase inhibition by methotrexate and TP. 72-hr cultures of Con A-stimulated lymphocytes preincubated with varying concentrations of 2-deoxyuridine for 1 hr followed by 1 hr incubation with 1×10^{-6} M methotrexate (○——○), 1×10^{-4} M TP (●——●) or drug diluent (▲——▲). 3-hr pulse of 1 μCi [^{3}H]thymidine given and cells extracted as before. [^{3}H]thymidine incorporation expressed as mean ± S.E.M. percentage of incorporation by control cultures pulsed without pre-incubation with deoxyuridine or drugs. All points on methotrexate curve differ significantly from control; only incorporation at lowest deoxyuridine concentration is significant for the TP-treated cultures.

Table 1. Effect of TP on [^{3}H]folic acid uptake at 72 hr by Con A transformed lymphocytes

Drug	Concentration	% of control folic acid uptake* Mean	S.E.M.	n	P†
Trimethoprim	5×10^{-4}	87.6	4.8	5	N.S.
	1×10^{-4}	92.3	3.0	11	N.S.
	5×10^{-5}	93.6	5.4	6	N.S.
Methotrexate	1×10^{-4}	81.0	5.7	5	0.03

* Control uptake = 23.2 pg/10^6 cells (S.E.M. 3.6; $n = 8$); without Con A uptake = 14.8 pg/10^6 cells (S.E.M. 3.2; $n = 7$).

† $P > 0.05$ = Not significant = N.S.

were added in various concentrations to lymphocytes transformed by Con A in the presence of 1×10^{-4} M TP, only minor and insignificant reversal of the TP-induced inhibition of [^{3}H]thymidine incorporation at 72 hr occurred. Thus under these conditions in the absence of folic acid 1×10^{-4} M TP reduced [^{3}H] thymidine incorporation by 42.5 ± 2.5% (S.E.M., $n = 12$). Adding 100 ng/ml folic acid to the medium reduced this inhibition to 12.4 ± 2.4% (S.E.M., $n = 9$) which was a significant reversal. Additions of 50, 400, 50000 and 100000 ng/ml folic acid however did not produce significant reversal. Additions of concentrations of folinic acid ranging from 30 to 60000 ng/ml failed to significantly alter the degree of inhibition of [^{3}H]thymidine incorporation produced by 1×10^{-4} M TP. Taken together these results suggest that there is no significant inhibition of human lymphocyte dihydrofolate reductase by TP at this concentration.

Effect of TP on blast formation and thymidine labelling. To investigate whether TP inhibited the process of transformation rather than thymidine uptake autoradiographic studies were undertaken. Table 2 shows that whilst TP did not affect the percentage of cells undergoing blastic transformation morphologically, the percentage of blasts labelled with thymidine as an indicator of DNA synthesis was reduced.

DISCUSSION

We have demonstrated that TP inhibits Con A-induced thymidine incorporation by human lymphocytes. Not only thymidine, but also uridine, leucine and formate incorporation were inhibited by TP in a dose-dependent fashion. This implies that TP is not acting to inhibit specific membrane transport of thymidine and suggests it may act upon some central metabolic function during transformation. The different effects seen on protein synthesis were of interest: overall protein synthesis reaches a peak at around 48 hr after the addition of mitogen [8] yet TP produces a lesser effect at this time compared to its effect when protein synthesis is waning at 72 hr. Protein synthesised during the period of maximal DNA synthesis (at 72 hr) is apparently more susceptible to TP inhibition, perhaps indicating that this effect is secondary to an effect on nucleic acid metabolism.

TP inhibition was also observed when lymphocyte transformation was induced in a mixed lymphocyte reaction suggesting that the inhibition is not dependent upon interference with Con A recognition sites. Inhibition of thymidine incorporation occurred even when TP was introduced many hours after the mitogenic stimulus, which again suggests a more complex mechanism than simple competition for mitogen binding sites since it is known that lymphocytes are irreversibly committed towards DNA synthesis after 6 hr incubation with phytohaemagglutinin [9].

These effects do not seem to be merely an apparent inhibition of incorporation due to non-specific killing of the cells by TP since no gross signs of toxicity were apparent as measured by Trypan Blue exclusion or changes in cell number. Neither can the effect be attributed to a more subtle derangement of lymphocyte function without gross changes in membrane permeability or cell death since the capacity of lymphocytes to respond to Con A was found to be unimpaired after pre-incubation with TMP.

The known inhibitory effects of TP on dihydrofolate reductase made this an obvious first choice for any investigation of the mechanism of action of TP. Studies on the human hepatic enzyme have shown that 50 per cent inhibition occurs at 3×10^{-4} M TP [10]. TP produces its effects on lymphocyte transformation at concentrations one order of magnitude smaller than this. It may be that the drug is selectively concentrated by lymphocytes or alternatively the lymphocyte enzyme could possess different kinetic properties. Indirect estimation of dihydrofolate reductase activity by the deoxyuridine titration method of Metz *et al.* [4] showed only minimal evidence of inhibition. It is interesting that diphenylhydantoin, another drug showing some immunosuppressive properties, demonstrates a similar phenomenon [11]. This technique depends upon a number of factors which include deoxyuridine uptake and phosphorylation, thymidine kinase activity, the activity of DNA salvage pathways and the rate of DNA synthesis. It could

Table 2. Effect of TP on blastic transformation and [^{3}H]thymidine labelling after 72 hr stimulation with Con A

	Percentage of cells undergoing transformation Mean	S.E.M.	n	P	Percentage of blasts labelled by ^{3}H thymidine Mean	S.E.M.	n	P
Control	82.5	1.8	6	—	10.4	0.5	6	—
TP 5×10^{-4} M	81.8	3.0	6	N.S.	4.3	0.6	6	0.001
TP 1×10^{-4} M	82.5	2.6	6	N.S.	4.7	0.8	6	0.001
TP 1×10^{-5} M	76.2	2.5	4	0.03	7.1	0.9	4	0.001

P value for comparison of drug-treated cultures with control (N.S. = Not Significant at $P < 0.05$).

not reveal dihydrofolate reductase inhibition if TP affected one of these processes. A small inhibitory effect on dihydrofolate reductase has been noted at 10^{-4} M TP [12, 13] although another study only detected inhibition at 10^{-1} M [14]. We have been unable to detect inhibition of the reductase of human fibroblasts in tissue culture undergoing epidermal growth factor-induced mitosis [15]. Although, like MTX and pyrimethamine, TP lowers the intracellular thymidine triphosphate content of human lymphocytes significant changes do not occur below 10^{-3} M [27].

Addition of folate or folinate (5-formyl tetrahydrofolate) only partially reverses the effects of TP on thymidine incorporation. Folate would act competitively with TP for dihydrofolate reductase whilst folinate would supplement the putatively depleted intracellular reduced folate pool. Two separate folate transport systems exist in lymphocytes: MTX shares the carrier for 5-methyl and 5-formyl tetrahydrofolate whilst folate enters by a separate mechanism [16, 17]. The effects of TP on folate transport in lymphocytes are unknown but our experiments with labelled folate make it unlikely that it significantly alters the access of folate at the drug concentrations employed. In human lymphoblastoid cell lines in culture TP appears to be a weak inhibitor of folate transport [18]. Interpretation of folate and folinate addition experiments is complicated by the inhibitory effects of these substances upon thymidine uptake. Bain [19] found that folic acid increased thymidine uptake in mixed lymphocyte reactions but decreased that of phytohaemagglutinin-treated lymphocytes although there was no effect on the number or percentage of blast cells formed. A possible explanation of this phenomenon is that in medium containing no folate, the intracellular folate stores plus folate released by dead cells allows DNA synthesis to proceed, although the intracellular thymidine pool is smaller due to reduced availability of folate coenzyme for its synthesis. There is thus less dilution of added [^{3}H]thymidine by endogenous thymidine and the specific activity of DNA synthesised from the pool is high. Adding increased amounts of folate would allow expansion of the thymidine pool thereby decreasing the specific activity of DNA synthesised after addition of exogenous [^{3}H]thymidine.

One of the principal actions of MTX is the inhibition of dihydrofolate reductase yet, unlike TP, the incorporation of [^{3}H]thymidine is apparently increased. Inhibition of dihydrofolate reductase depletes the supply of reduced methylene tetrahydrofolate which is required for conversion of deoxyuridylate to thymidylate. The reduced size of the intracellular thymidine pool will, as explained above, increase the specific activity of DNA synthesised after [^{3}H]thymidine addition although radiophosphorus labelling shows DNA synthesis to be reduced by MTX [20].

Inhibition of thymidine uptake by TP could indicate either that in the presence of the drug a reduced number of cells respond to the mitogenic stimulus or that the same number of cells respond but the amount of thymidine incorporated is reduced. Autoradiographic studies were undertaken to distinguish between these possibilities. TP did not alter the number of cells undergoing morphological transformation although the incorporation of [^{3}H]thymidine into their DNA is significantly reduced. Salzmann *et al.* [21] have similarly shown that if DNA synthesis is blocked by 5-fluoro-2'-deoxyuridine the transformation of lymphocytes is unaffected. Likewise, Rozenszajn and Radnay [22] found that mitosis is 1000-fold more sensitive to MTX than is transformation. Dipyramidole at a concentration twenty times that required to completely inhibit uridine membrane transport with secondary inhibition of RNA synthesis also does not alter the percentage of cells transformed [23]. These observations suggest that interruption of the cell cycle at a phase of DNA synthesis is not an unusual mode of drug action and is produced by several different mechanisms.

It is tempting to speculate upon the clinical significance of the phenomena described. The lymphocyte blastogenic response to Con A represents an *in vitro* model for the early stages of cellular immunity. TP plasma levels during therapy are approximately 10^{-5} M [24] a level at which we observed approximately 50 per cent inhibition in the mixed lymphocyte reaction, although less inhibition was seen in lectin transformed cells. TP is 42–46% protein bound [25] and concentration in tissues is known to occur [26]. The *in vitro* phenomenon described could be associated with the immunosuppressive effects observed by other investigators. Possibly caution should be exercised in the interpretation of clinical tests dependent upon lymphocyte transformation when the patient is concurrently receiving TP.

We conclude that TP suppresses some of the metabolic consequences of lymphocyte transformation although the morphological features of the process are unaffected. We have found no evidence that this effect is dependent on inhibition of dihydrofolate reductase and there are indications that a second mechanism, perhaps involving nucleic acid metabolism is implicated.

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