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INHIBITION OF LYMPHOCYTE NUCLEIC ACID METABOLISM AND ANTIBODY PRODUCTION BY TRIMETREXATE*

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Abstract—Trimetrexate is a lipid soluble dihydrofolate reductase inhibitor which, unlike methotrexate, does not depend upon the membrane folate transport system for cell entry. We investigated the possibility that trimetrexate (but not methotrexate) might permeate intermitotic lymphocytes and, following stimulation, impair only the responding cells, rather than all dividing cells, as is the case with methotrexate. Peripheral blood mononuclear cells from normal individuals were incubated for 1 hr in three moderate to high concentrations (1, 10 and 100 μM) of methotrexate or trimetrexate, washed, and incubated with phytohemagglutinin. Intracellular folate activity, as assessed by the deoxyuridine suppression test, was abnormal at all three concentrations of trimetrexate but only at the highest concentration of methotrexate. Similarly, incorporation of [^{3}H]deoxyuridine was depressed profoundly in trimetrexate-treated cells (2% of control) but unaffected by methotrexate. Analysis of cell cycle distribution by flow cytometry confirmed $G_0 + G_1$ arrest in trimetrexate but not methotrexate-treated cells. Neither drug altered morphologic transformation, Tac antigen expression, or incorporation of [^{3}H]thymidine by the "salvage" pathway. Therefore, brief exposure to methotrexate has little effect on intermitotic lymphocytes, whereas trimetrexate very specifically inhibits the conversion of deoxyuridine to thymidine in these cells and leads to the arrest of DNA synthesis in the $G_0 + G_1$ phase. This metabolic abnormality markedly reduces *in vitro* antibody synthesis: a 1-hr treatment of lymphocytes with 10 or 100 μM trimetrexate prior to incubation with pokeweed mitogen on four occasions completely inhibited both IgG and IgM secretion. Similar treatment with methotrexate had no effect until the highest concentration (100 μM) was used. We conclude that brief exposure of peripheral blood mononuclear cells to the nonclassical dihydrofolate reductase inhibitor, trimetrexate, results in inhibition of nucleic acid synthesis and impairment of antibody production. This drug effect may permit more incisive modulation of immune responses.

Cytotoxic drugs are used extensively in clinical practice to moderate or inhibit immune responses [1]. Experimental studies have shown that these immunosuppressive agents are most effective when administered contemporaneously with an inciting antigen [2–4]. While the pharmacologic basis for this requirement has not been elucidated entirely, it is known that some of these drugs, such as 6-mercaptopurine, methotrexate and cytosine arabinoside, are cycle active, whereas others, like cyclophosphamide, may affect antigen processing and early "information" transfer [3–5]. Thus, they are most effective against newly stimulated, dividing lymphocytes and have less effect on either intermitotic or post-stimulation cells. Optimally, then, drug administration should be timed to coincide with antigenic stimulation. Unfortunately in most clinical situations the inciting antigen is unknown. Consequently, most immunosuppressive agents must be administered chronically in order to be effective. This therapeutic approach has at least two important disadvantages. Drug effect is relatively non-specific in that all dividing cells are affected. As a result, side-effects are frequent and sometimes severe [6]. Second, many immunosuppressive agents when used chronically are associated with an increased risk of malignancy, particularly leukemia [6, 7].

Development of a lipid-soluble dihydrofolate reductase (DHFR‡) inhibitor, trimetrexate, 6([(3,4,5 - trimethoxyphenyl)anilino]methyl) - 5 - methyl 2,4-quinazolinediamine (TMQ), offers the possibility of an immunosuppressive agent which circumvents many of these problems (Fig. 1). The classic DHFR inhibitor, methotrexate (MTX), has gained widespread acceptance in clinical practice because it has rarely been associated with subsequent

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‡ Abbreviations: DHFR, dihydrofolate reductase; TMQ, trimetrexate; MTX, methotrexate; PBMNCs, peripheral blood mononuclear cells; DMEM, Dulbecco's Modified Eagle's Medium; PHA, phytohemagglutinin; [^{3}H]dU, tritiated deoxyuridine; [^{3}H]TdR, tritiated thymidine; PBS, phosphate-buffered saline; and PWM, pokeweed mitogen.

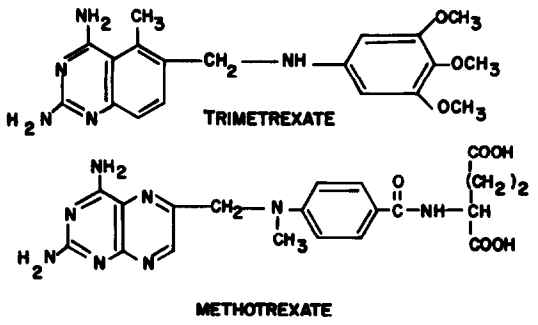


Fig. 1. Structural formulas of trimetrexate and methotrexate.

development of malignancies [7]. However, methotrexate, like its physiologic counterpart, 5-methyltetrahydrofolic acid, is transported into lymphocytes only when they are activated [8, 9]. Consequently, it too must be administered chronically in anticipation of antigenic stimulation. Since trimetrexate incorporation is independent of the folate membrane transport system, we postulated that this new lipid-soluble agent might also permeate into intermitotic lymphocytes and bind to their DHFR. As a result, these cells would be limited in their response to antigen. In this way, selectivity might be enhanced, since only immunologically activated lymphocytes would be impaired. Moreover, the drug could be given on a single or intermittent high dose schedule to "load" the cells, thereby obviating the disadvantages of chronic administration. Finally, since trimetrexate is not subject to polyglutamation, it may have a different toxicity profile than methotrexate. For these reasons, we studied the effects of TMQ on lymphocyte stimulation and response.

MATERIALS AND METHODS

Isolation of mononuclear cells. Human peripheral blood mononuclear cells (PBMNCs) were isolated from healthy volunteers by Ficoll–Hypaque density gradient centrifugation (Histopaque-1077; Sigma Diagnostics). PBMNCs were resuspended at a concentration of 1×10^6 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) (Whittaker M. A. Bioproducts, Walkersville, MD), supplemented with 2% glutamine, 1% penicillin-streptomycin, and 10% dialyzed horse serum (all obtained from Gibco Laboratories, Grand Island, NY). In some experiments, PBMNCs were grown in "folate-free" Dulbecco's Modified Eagle's Medium which contained 0.26 ng/ml of folate activity by the *Lactobacillus casei* assay [9, 10]. Under similar experimental conditions, others have reported mean intra-lymphocyte folate content in supplemented and folate deficient media of 404 pg/10^6 cells and 135 pg/10^6 cells respectively [11]. For assays of immunoglobulin synthesis, PBMNCs were cultured in RPMI 1640 containing penicillin-streptomycin and 10% fetal bovine serum (Gibco Laboratories).

Drug preparation. Liquid, preservative-free methotrexate (MTX) (Lederle Laboratories, Pearl River, NY) was diluted in 0.9% NaCl solution, and a 10 mM stock solution was prepared. A 10 mM stock solution of trimetrexate glucuronate (TMQ), a gift from the Warner-Lambert Co. (Ann Arbor, MI) was prepared using deionized water.

For 1-hr incubations, stock solutions of MTX and TMQ were diluted further in NaCl and water respectively. After sterile filtration, 10 μl drug/ml of cells was added to give final drug concentrations of 100, 10 and 1 μM for each drug. For 72-hr incubations final concentrations of 1 μM were used. Stock solutions were prepared fresh on the day of the experiment.

Experimental design. For 1-hr incubations, 5 ml of cell suspension was placed in sterile 17 × 100 mm culture tubes (American Scientific Products). MTX and TMQ were added to achieve 100, 10 and 1 μM final concentrations as previously described. An equal volume of 0.9% NaCl was added to the control tubes. Tubes were incubated at 37° with 5% CO_2, with gentle agitation at 15-min intervals. After 1 hr, tubes were centrifuged (850 g × 10 min), and PBMNCs were washed twice with DMEM and then resuspended to the original volume.

The 72-hr incubations were prepared in a similar manner, using 1 μM drug concentrations and omitting the washing step. Cells for culture in low folate medium were centrifuged, washed twice in "folate-free" DMEM, and resuspended to their original volume in this medium. No drug was added to these cells.

Following drug exposure or suspension in low folate medium, cells (100 μl) were plated in quadruplicate in microtitration multi-well plates (Nunclon 96-well plates). For controls without PHA, 100 μl DMEM was added to each well. For mitogen-stimulated cells, 20 μl of a 0.025% PHA solution (Bacto Phytohemagglutinin P; DIFCO, Detroit, MI) was added to each well with enough medium to bring the total volume to 200 μl.

Plates were incubated at 37° with 5% CO_2 for 72 hr, and the following studies were performed.

Measurement of [^{3}H]*dU uptake.* At 18–24 hr prior to harvest, 10 μl containing 0.4 μCi [^{3}H]deoxyuridine (21.4 Ci/mmol) (DuPont, New Research Products, Boston, MA) was added to all wells: with and without mitogen, 1- and 72-hr incubations, control cells, and cells cultured in low folate medium. Cells were harvested with a mini-MASH (M. A. Bioproducts, Maryland) cell harvester, and radioactivity was counted.

Incorporation of [^{3}H]*thymidine and deoxyuridine suppression test.* Four hours prior to harvest, 20 μl of a solution containing 1 mM deoxyuridine was added to wells with and without PHA. Phosphate-buffered saline [9] (20 μl) was added to additional wells containing PHA-stimulated cells (control cells). After a 1-hr incubation, 10 μl containing 0.4 μCi [*methyl*-^{3}H]thymidine (20 Ci/mmol; CEA, France) was added to all wells, and cells were harvested 3 hr later. Deoxyuridine suppression was measured as a ratio of [^{3}H]TdR incorporation into stimulated cells plus deoxyuridine to stimulated cells plus PBS, expressed as a percentage.

Flow cytometry analysis. After 96 hr of incubation, 1×10^6 cells from mitogen-stimulated control, drug-exposed and low folate cultures were centrifuged and

stained using a slight modification of the technique of Krishan [12]. Cells were suspended in a hypotonic 0.1% sodium citrate solution containing 50 μg/ml propidium iodide (Sigma), 500 μg/ml RNAase (Sigma), 0.05% NP40 nonionic detergent (Sigma), and 10% horse serum. Tubes were incubated in a 37° water bath for 30 min prior to instrumentation: 50-H/H Cytofluorograf flow cytometer, interfaced with an Ortho 2150 data handling system (Ortho Diagnostics Systems, Inc., Westwood, MA). Excitation was by argon laser, operating in output stabilized mode at an output of 200 MW, wavelength 488 nm. Red fluorescence was defined by a 560 nm short pass dichroic (Omega Optics Inc., Brattleboro, VT) and a 630 nm long pass filter (Schott). By correlating the integrated area with the peak of each fluorescence pulse in a two parameter cytogram, a rectilinear region could be set which excluded signals due to nuclear doublets from further analysis. Fluorescence of cells falling in this defined region was then presented as a one parameter histogram of red fluorescence. Typically, 50,000 events were acquired per histogram.

Anti-Tac monoclonal antibody. After 72 hr of incubation, tubes containing 1×10^6 stimulated and unstimulated cells from control groups and 72-hr drug-exposed (1 μM) groups were spun, and all but 50 μl supernatant fraction was removed. Ten microliters of anti-Tac antibody (provided by Dr. T. Waldmann, NIH, Bethesda, MD) and 40 μl PBS with 0.1% azide were added to half of the tubes followed by incubation for 30 min at 4°. Cells were washed once in cold PBS with 0.1% azide and again with cold medium. After centrifugation, all but 50 μl of the supernatant fraction was removed. Ten microliters of goat antimouse Ig (FITC conjugated IgG) (1:50 concn), was added to tubes containing anti-Tac antibody as well as to controls. Tubes were incubated for 30 min at 4° and washed with PBS with azide. Cells were spun again and resuspended in 1 ml PBS.

The percent positive cells was calculated by subtracting the number of cells reactive with non-specific antibody from the number reactive with specific antibody.

Immunoglobulin synthesis. Mitogen-induced immunoglobulin (Ig) synthesis was determined as previously described [13]. In brief, 10^6 PBMNCs were cultured in 2 ml of complete medium with and without pokeweed mitogen (PWM) diluted 1:200. An enzyme-linked immunosorbent assay (ELISA) was used to measure the IgG and IgM in the culture supernatant fractions after 12 days in culture.

Uptake studies. PBMNCs were isolated as described above and cultured for 96 hr with or without PHA. L-Glutamyl[3,4-^{3}H]methotrexate (41.1 Ci/mmol; New England Nuclear, Boston, MA) or [^{14}C]TMQ (9.5 mCi/mmol; obtained from Dr. Robert Engel, National Cancer Institute, Bethesda, MD) was added as a final concentration of 10 μM to approximately 2×10^6 cells in 1 ml of culture medium. Cells were harvested immediately and after a 1-hr incubation by centrifuging for 45 sec at 1000 *g*, washing once with ice-cold PBS, and filtering with suction (Whatman GF/A, Maidstone, England). Radioactivity was determined with a scintillation counter. Uptake was calculated by subtracting initial sample radioactivity from 1-hr sample radioactivity to correct for non-specific binding and trapping of isotope.

Table 1. Effects of different exposure times and concentrations of trimetrexate and methotrexate on the deoxyuridine suppression of tritiated thymidine ([^{3}H]TdR) incorporation by PHA-stimulated PBMNCs

Treatment of cells	% of Control*	P†	N‡
None	4.1 ± 0.6		10
Low folate medium	20.1 ± 3.9	<0.025	4
Trimetrexate			
1 μM (72 hr§)	33.2 ± 3.2	<0.001	5
1 μM (1 hr)	14.6 ± 4.4	<0.05	10
10 μM (1 hr)	37.5 ± 5.8	<0.005	5
100 μM (1 hr)	30.8 ± 3.6	<0.005	5
Methotrexate			
1 μM (72 hr)	31.1 ± 3.4	<0.005	5
1 μM (1 hr)	5.5 ± 2.8	NS‖	10
10 μM (1 hr)	5.2 ± 1.0	NS	5
100 μM (1 hr)	15.8 ± 4.2	<0.05	5

* Control (100%) is [^{3}H]TdR uptake by cells without added deoxyuridine (see Table 4 for absolute values). Values are means ± SEM.

† P values were determined by the paired *t*-test comparing percent suppression in treated cells with parallel cultures of untreated cells.

‡ Number of experiments, each performed in at least triplicate.

§ Duration of exposure to drug.

‖ Not significant.

Statistics. All data except that in Table 5 were analyzed using the paired *t*-test. Data in Table 5 were analyzed with Wilcoxon's Rank Sum test.

RESULTS

The incorporation of tritiated thymidine ([^{3}H]TdR) into DNA by mitogen-stimulated lymphocytes was decreased more than 95% after a 1-hr incubation with 1 mM deoxyuridine (Table 1). Deoxyuridine is converted to thymidylate in a folate-dependent step; consequently little exogenous [^{3}H]TdR is incorporated by the "salvage" pathway (Fig. 2). However, if the cells have decreased intra-

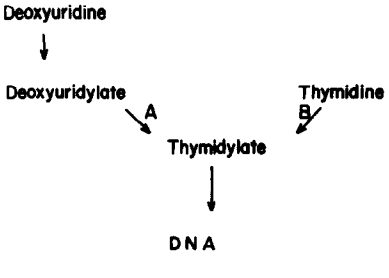


Fig. 2. Relationship of folate metabolism to DNA synthesis.

Table 2. Tritiated deoxyuridine ([^{3}H]dU) incorporation by PBMNCs after exposure to trimetrexate or methotrexate for 1 or 72 hr

Treatment of cells	[^{3}H]dU incorporation (cpm) +PHA	P*	N†	[^{3}H]dU incorporation (cpm) No PHA	P	N
None	46,500 ± 5,000‡		11	530 ± 80		11
Low folate medium	47,000 ± 6,700	NS§	5	580 ± 100	NS	5
Trimetrexate						
1 μM (72 hr‖)	2,000 ± 710	<0.001	6	530 ± 100	NS	6
1 μM (1 hr)	45,800 ± 5,600	NS	10	500 ± 70	NS	9
10 μM (1 hr)	16,300 ± 4,800	<0.001	5	1,100 ± 540	NS	5
100 μM (1 hr)	750 ± 170	<0.005	5	820 ± 380	NS	5
Methotrexate						
1 μM (72 hr)	240 ± 60	<0.001	6	450 ± 80	NS	6
1 μM (1 hr)	42,600 ± 4,900	NS	11	470 ± 50	NS	11
10 μM (1 hr)	31,400 ± 4,800	NS	4	580 ± 120	NS	5
100 μM (1 hr)	33,000 ± 5,100	NS	5	590 ± 40	NS	5

* P values were determined by the paired *t*-test comparing [^{3}H]dU uptake by treated and untreated cells in parallel cultures.
† Number of experiments, each performed in at least triplicate.
‡ Mean ± SEM.
§ Not significant.
‖ Duration of exposure to drug.

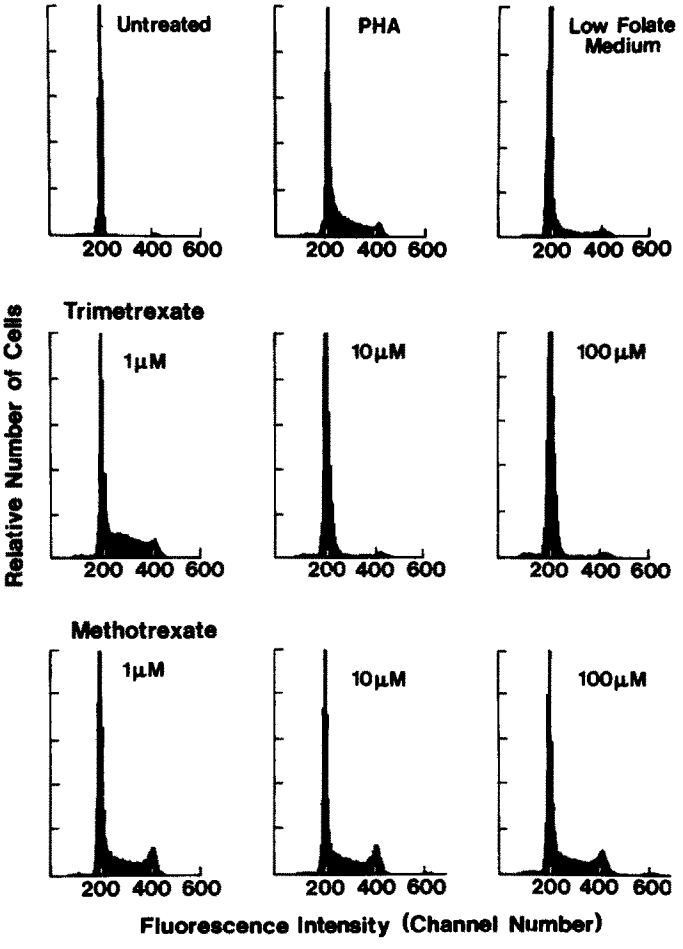


Fig. 3. DNA per cell distribution histograms of PBMNCs treated with folate antagonists. Fluorescence intensity is proportional to DNA content. Numbers on the abscissa indicate channel numbers. Some PBMNCs were treated with trimetrexate or methotrexate in indicated concentrations for 1 hr prior to incubation with PHA. The first peak, at about channel 200, represents $G_0 + G_1$ cell DNA content, while the peak at about 400 represents $G_2 + M$. The plateau between the two peaks represents S DNA content.

cellular folate activity, less deoxyuridylate is converted to endogenous thymidylate and [^{3}H]TdR incorporation into DNA increases. Thus, the deoxyuridine suppression test is a sensitive index of folate availability.

As expected, PHA-stimulated lymphocytes incubated for 72 hr in folate-deficient medium manifested impaired suppression of thymidine uptake. These folate-deficient cells were found to have a thymidine incorporation of approximately 20% of control cell cultures without added deoxyuridine. In comparison, cells in standard medium incorporated less than 5% of control cultures. Seventy-two-hour incubations with the DHFR inhibitors MTX or TMQ in 1 μM concentrations resulted in subnormal suppression (Table 1). In parallel experiments, some cells were treated for only 1 hr with MTX or TMQ in three concentrations, washed, and then placed in culture with PHA for 72 hr. Deoxyuridine suppression tests now showed statistically significant abnormalities of folate activity at all three concentrations of TMQ but only at the highest concentration of MTX (Table 1). Interestingly, the extent of suppression after treatment with 100 μM MTX was similar to that observed with 1 μM TMQ, whereas impaired suppression was much more evident at the higher concentrations of TMQ. These results suggest an approximate 2 log difference in the capacity of these drugs in favor of TMQ to inhibit folate activity in unstimulated lymphocytes.

This interpretation is supported by uptake studies with radiolabeled TMQ and MTX. Unstimulated lymphocytes incorporated approximately 67 pmol/10^6 cells (range 46–82) of TMQ during a 1-hr incubation, but only 0.18 pmol/10^6 cells (0–0.36) of MTX. The results with mitogen-stimulated cells were similar: uptake of 90 pmol/10^6 cells (65–118) of TMQ and 0.88 pmol/10^6 cells (0.16–16) of MTX.

Drug effects were tested more directly by measuring incorporation of tritiated deoxyuridine ([^{3}H]dU) into DNA. Incubation of PHA-stimulated lymphocytes with either 1 μM MTX or 1 μM TMQ for 72 hr predictably decreased incorporation of [^{3}H]dU (Table 2). However, this assay is a less sensitive index of low folate activity than the deoxyuridine suppression test, since incorporation of [^{3}H]dU by cells in low folate medium was not reduced significantly compared to cells in standard medium. Nevertheless, brief exposure to TMQ prior to mitogen stimulation profoundly depressed [^{3}H]dU incorporation (more than 98% at 100 μM), while MTX in all concentrations had a slight but not statistically significant inhibitory effect.

As a third measure of drug effects, cell cycle distribution was determined by flow cytometry. Histograms from a representative experiment are shown in Fig. 3, and statistical analyses of multiple experiments are presented in Table 3. Measurement of the cell cycle distribution of unstimulated lymphocytes indicated that most of the DNA was in the $G_0 + G_1$ phase (2C), whereas PHA stimulated lymphocytes had a higher percentage of cells with DNA content in S phase or $G_2 + M$ (4C). After a 72-hr incubation in either folate-deficient medium or medium containing anti-fols, DNA synthesis was arrested in $G_0 + G_1$ (Table 3). Exposure of lymphocytes for 1 hr to MTX in the concentrations tested prior to mitogen stimulation had no effect on cell cycle distribution. In contrast, TMQ at a 10 μM concentration reduced the number of cells with a 4C DNA content and, at 100 μM, nearly all DNA was arrested at 2C.

Although both prolonged exposure to MTX or TMQ and brief treatment with TMQ profoundly affected folate activity and DNA synthesis, these drugs did not prevent lymphocyte stimulation under the conditions of these experiments. Morphologically, the drug-treated cells were identical to mitogen-stimulated cells in standard medium (Fig. 4), and Tac antigen expression was similar on mitogen-stimulated lymphocytes when measured after 72-hr incubations with MTX, TMQ or saline added to the suspending medium (Fig. 5). Incorporation of [^{3}H]TdR by the salvage pathway was intact, and thymidine uptake was comparable in drug-treated or folate-deficient mitogen-stimulated cells to control cells (Table 4). Therefore, under the conditions of

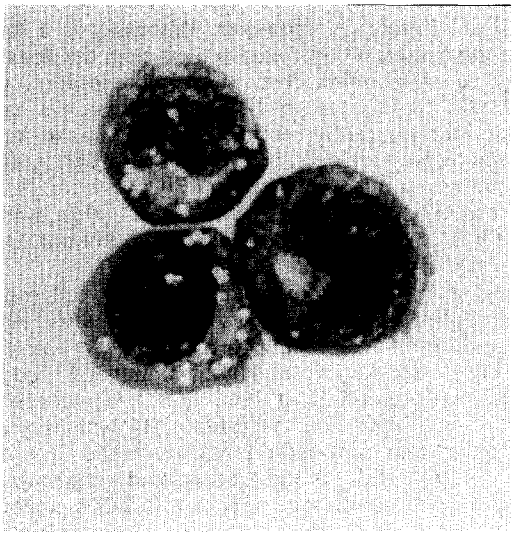
Table 3. Cell cycle distribution of human lymphocytes after treatment with anti-folates

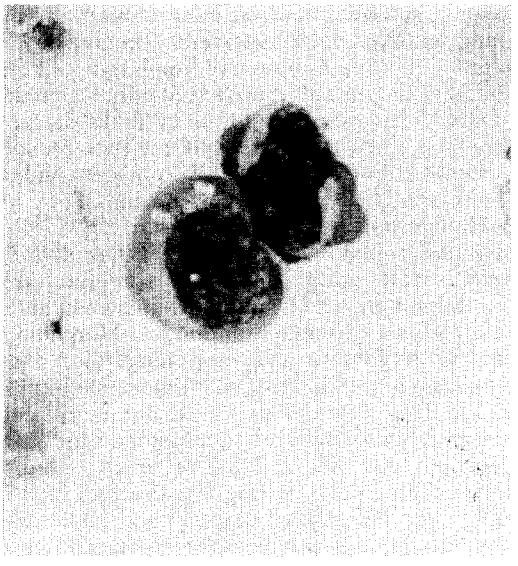
Treatment	$G_0 + G_1$	S	$G_2 + M$	N*
None	99.0 ± 1.0†‡	1.2 ± 0.3‡	1.0 ± 0.1§	12
PHA	81.1 ± 3.2	10.9 ± 2.1	8.0 ± 1.9	12
Low folate medium	88.2 ± 5.0‖	6.1 ± 2.5	7.2 ± 1.9	7
Trimetrexate				
1 μM (72 hr)	99.0 ± 0.3¶	0.3 ± 0.3¶	1.2 ± 0.4	4
1 μM (1 hr)	75.6 ± 3.8	16.5 ± 3.3	8.0 ± 1.5	10
10 μM (1 hr)	89.5 ± 4.3	10.0 ± 3.5	3.1 ± 0.3‡	6
100 μM (1 hr)	98.7 ± 1.0‡	0.8 ± 0.4‡	1.5 ± 0.3‡	6
Methotrexate				
1 μM (72 hr)	95.1 ± 1.9‖	3.2 ± 1.7	1.7 ± 0.3	4
1 μM (1 hr)	80.2 ± 2.5	10.0 ± 1.9	9.9 ± 1.3	10
10 μM (1 hr)	79.7 ± 4.5	14.0 ± 4.1	6.4 ± 1.2	6
100 μM (1 hr)	87.1 ± 6.5	10.6 ± 4.6	4.8 ± 1.5	6

* Number of experiments.

† Mean ± SEM.

‡–¶ Significantly different relative to PHA-treated cells: ‡ $P < 0.001$, § $P < 0.005$, ‖ $P < 0.05$ and ¶ $P < 0.025$.





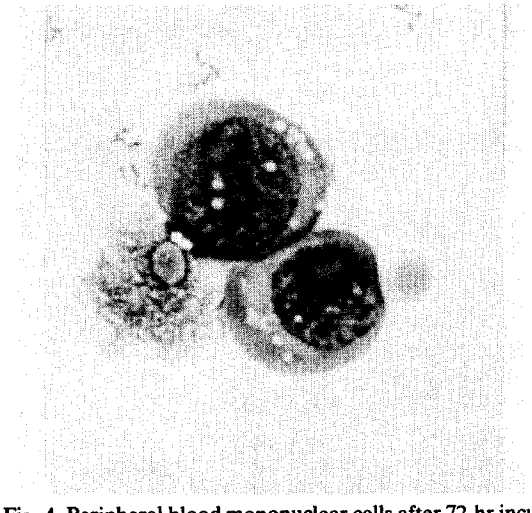


Fig. 4. Peripheral blood mononuclear cells after 72-hr incubations with PHA and culture medium alone (top), 1 μM trimetrexate (centre), or 1 μM methotrexate (bottom). The DHFR inhibitors did not block morphologic transformation in this concentration. (Light microscopy, Wright's stain, original magnification ×1000.)

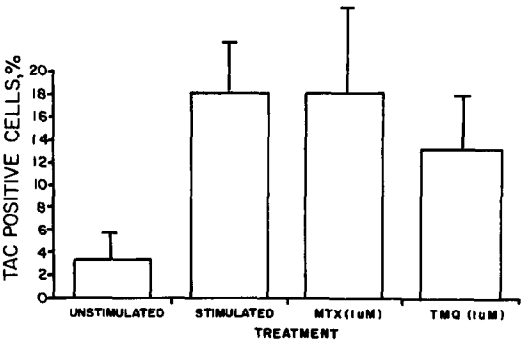


Fig. 5. Percentage of Tac^+ cells in unstimulated cultures and in cultures with added PHA. Methotrexate (MTX) or trimetrexate (TMQ) in 1 μM concentrations was added to some PHA-stimulated cultures. Brackets indicate SEM.

these experiments, MTX and TMQ did not cause extensive cytolysis. The data do not exclude the possible loss of a subpopulation of cells, however.

Taken together, these studies indicate that brief exposure to TMQ but not MTX inhibited folate activity and DNA synthesis in lymphocytes but did not cause major cytolysis or interfere with their capacity to respond morphologically to mitogen. We were interested in determining whether this metabolic abnormality would influence an important lymphocyte function, such as immunoglobulin production. As shown in Table 5, a 1-hr treatment of lymphocytes with 10 or 100 μM TMQ prior to incubation with pokeweed mitogen on four occasions completely inhibited both IgG and IgM secretion into the suspending medium. Similar treatment with MTX had no effect until the highest concentration (100 μM) was used.

DISCUSSION

Deficiency of folic acid or pharmacologic inhibition of DHFR depletes intracellular levels of 5–10 methylene tetrahydrofolate. This, in turn, decreases the use of deoxyuridine for DNA–thymine synthesis. Moderate depletion of the tetrahydrofolate pool results in impaired deoxyuridine suppression of exogenous thymidine uptake, decreased incorporation of deoxyuridine into DNA, and accumulation of DNA synthesis in $G_0 + G_1$ and S phases [8, 14–16]. More profound folate depletion causes cell death by impairing both purine and pyrimidine synthesis [15, 16]. The present studies with human peripheral blood lymphocytes exemplify these effects of altered folate metabolism on DNA synthesis. Incubation of PBMNCs with PHA for 72 hr in either folate-deficient medium or in medium containing DHFR inhibitors (MTX or TMQ) interfered with *de novo* DNA–thymine synthesis from deoxyuridine and led to accumulation of DNA in the $G_0 + G_1$ phase. These incubation conditions were not lethal for most of the cells, however, since thymidine incorporation by the "salvage" pathway was intact, and uptake of exogenous [^{3}H]TdR was not significantly different from that by control cells in standard culture medium.

Table 4. Effects of different exposure times and concentrations of trimetrexate and methotrexate on tritiated thymidine ([^{3}H]TdR) uptake by PBMNCs

Treatment of cells	[^{3}H]TdR uptake (cpm) +PHA	P*	N†	[^{3}H]TdR uptake (cpm) No PHA	P	N
None	31,800 ± 5,900‡		11	410 ± 60		11
Low folate medium	25,600 ± 8,400	NS§	4	200 ± 60	<0.05	5
Trimetrexate						
1 μM (72 hr‖)	23,800 ± 5,300	NS	5	160 ± 60	<0.05	6
1 μM (1 hr)	22,800 ± 2,900	NS	11	310 ± 50	NS	11
10 μM (1 hr)	28,800 ± 5,400	NS	6	260 ± 100	NS	6
100 μM (1 hr)	16,700 ± 2,000	NS	6	340 ± 110	NS	6
Methotrexate						
1 μM (72 hr)	25,300 ± 5,400	NS	5	210 ± 30	<0.025	6
1 μM (1 hr)	21,900 ± 4,100	NS	11	340 ± 60	NS	11
10 μM (1 hr)	18,800 ± 3,000	NS	6	330 ± 100	NS	6
100 μM (1 hr)	21,000 ± 3,700	NS	6	410 ± 100	NS	6

* P values were determined by the paired *t*-test comparing [^{3}H]TdR uptake by treated and untreated cells in parallel cultures.
† Number of experiments, each performed in at least triplicate.
‡ Mean ± SEM.
§ Not significant.
‖ Duration of exposure to drug.

Moreover, the folate-deficient cells are capable of responding to mitogen, as assessed by morphology and by expression of Tac antigen [17].

TMQ appeared to be approximately two logs more potent than MTX when the cells were exposed briefly to the drugs prior to incubation with mitogen. Abnormal deoxyuridine suppression was seen following a 1-hr exposure to all three concentrations (1, 10 and 100 μM) of TMQ, but only at the highest concentration of MTX. Similarly, [^{3}H]dU incorporation was decreased significantly by 10 and 100 μM TMQ but not by 100 μM MTX. Cell cycle distribution of DNA was altered significantly by 10 and 100 μM TMQ but not by MTX in any of these concentrations. These findings are consistent with our postulate that TMQ, because of its lipid solubility, can enter intermitotic lymphocytes, but that the same cells are relatively impermeable to MTX. This interpretation is supported by our observation that unstimulated lymphocytes incorporated more than 300 times as much TMQ as MTX during a 1-hr incubation. After mitogen stimulation, the TMQ-treated cells then evidenced impaired DNA synthesis while the MTX-treated cells did not. The biochemical explanation(s) for this inhibition by TMQ is unknown at present. It seems plausible, however, that at least part of the mechanism of action is related to DHFR inhibition, since human lymphocytes have measurable amounts of this enzyme activity when cultured under the conditions of our experiments [18].

Whether the degree of abnormal lymphocyte metabolism induced by the conditions of these experiments alters immune responses *in vivo* is unknown at present. Folic acid deficiency is associ-

Table 5. Effect of brief exposure to various concentrations of anti-folates on IgG and IgM production

Treatment of cells	IgG	P*	IgM	P
None†	5,040 (500–10,000)‡		6,814 (1,600–13,400)	
Trimetrexate				
1 μM	81 (48–192)	<0.02	0 (0–330)	<0.02
10 μM	0 (0)	<0.02	0 (0)	<0.05
100 μM	0 (0)	<0.05	0 (0)	<0.05
Methotrexate				
1 μM	6,560 (102–6,720)	NS§	5,950 (280–13,200)	NS
10 μM	5,680 (300–6,260)	NS	3,885 (1,160–11,600)	NS
100 μM	22 (0–182)	<0.05	0 (0–960)	<0.05

* P values were determined by Wilcoxon's rank sum test.
† Parallel cultures in the absence of pokeweed mitogen produced 52 ng/ml IgG (range 0–72 ng/ml) and no IgM.
‡ Median (range in ng/ml).
§ Not significant.

ated with defects of both cellular and humoral immunity [19]. Studies in animals indicate that methotrexate administration can inhibit primary and secondary immune responses, reduce the cutaneous response to purified protein derivative, and induce tolerance [20]. More recently, MTX has also been shown to inhibit *in vitro* immunoglobulin synthesis [21]. Our studies indicate that a 1-hr exposure to all concentrations of TMQ tested, but only to 100 μM MTX, profoundly decreased IgG and IgM production after pokeweed mitogen stimulation. Therefore, it appears that pulse TMQ therapy influences at least one important lymphocyte function. The effect of this drug schedule on other functions needs to be investigated.

TMQ affords several potential advantages over MTX and other cytotoxic drugs as an immunosuppressive agent, provided it can be shown to be safe and effective. Pre-clinical and Phase I trials of TMQ suggest that it is well tolerated and that drug concentrations approximating those used in our studies are readily achievable [22, 23]. It appears, therefore, that this novel new agent presents an opportunity to more incisively modulate immune responses.

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