Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes: a metabolic basis for efficacy in rheumatoid arthritis?

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The immunosuppressive and anti-inflammatory effects of low-dose methotrexate (MTX) have been related directly to inhibition of folate-dependent enzymes by polyglutamated derivatives, or indirectly to adenosine release and/or apoptosis and clonal deletion of activated peripheral blood lymphocytes in S-phase. In this study of phytohaemagglutinin-stimulated primary human T-lymphocytes we show that MTX (20 nM to 20 μ M) was cytostatic not cytotoxic, halting proliferation at G₁. This stasis of blastogenesis was associated with an inhibition of purine ribonucleotide synthesis but a stimulation of pyrimidine biosynthesis, the normal mitogen-induced expansion of ATP and GTP pools over 72 h being restricted to concentrations of unstimulated T-cells, whereas the increment in UTP pools exceeded that of controls. Decreased incorporation of H¹⁴CO₃⁻ or [¹⁴C]glycine into purine ribonucleotides, with no radiolabel accumulation in any *de novo* synthetic intermediate but enhanced H¹⁴CO₃⁻ incorporation into UTP, supported these MTX-related effects. Exaggerated [¹⁴C]hypoxanthine salvage (which normalized the purine and UTP pools) confirmed the increased availability of 5-phospho-

ribosyl-1-pyrophosphate (PP-ribose-P) as the molecular mechanism underlying these disparate changes. These results provide the first substantive evidence that the immunosuppressive effects of low-dose MTX in primary blasting human T-lymphocytes relate not to the inhibition of the two folate-dependent enzymes of purine biosynthesis but to inhibition of the first enzyme, amidophosphoribosyltransferase, thereby elevating PP-ribose-P and stimulating UTP synthesis. Varying cell types or incubation conditions employed by other workers, especially malignant/activated cells with high basal metabolic rates, might mask the effects noted in primary human T-lymphocytes. The findings imply the involvement of low-dose MTX in the inhibition of T-lymphocyte proliferation and proliferation-dependent processes in rheumatoid arthritis.

Key words: adenosine, amidophosphoribosyltransferase, 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate, leflunomide, phosphoribosylpyrophosphate.

INTRODUCTION

Methotrexate (MTX) is an immunosuppressive agent that has been in clinical use for over 40 years [1]. Although originally introduced for chemotherapy in cancer and leukaemia [1–7], MTX was coincidentally found to have immunosuppressive properties and is now the drug of choice in treating rheumatoid arthritis (reviewed in [8–11]). Early studies in malignant cells regarding the mode of action of MTX focused on its role as an antifolate [1]. The major target demonstrated for MTX was the inhibition of dihydrofolate reductase (DHFR). However, MTX-related effects were noted that involved both purine ribonucleotide (Scheme 1) and pyrimidine deoxyribonucleotide synthesis. These included inhibition at the level of the ninth folate-dependent step of purine synthesis catalysed by 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate (AICARibotide) transformylase (Scheme 1) and the pyrimidine

enzyme thymidylate synthetase [1]. Studies in human breast cancer cells questioned whether MTX was instead a prodrug, its mode of action resulting from the formation of polyglutamated derivatives of MTX and folates. The latter were retained within the cell, thereby exerting inhibitory effects on AICARibotide transformylase and thymidylate synthetase as well as on DHFR [7].

The mechanism by which MTX modulates inflammation in rheumatoid arthritis, particularly at the low-dose weekly regimes that have proved beneficial, is still debated [8–26]. A wide range of cellular and humoral effects have been cited to explain the anti-inflammatory properties of MTX. These include effects on both proliferation and antibody synthesis in peripheral blood mononuclear cells (PBMC) from rheumatoid arthritis patients or healthy humans [8–26]. Discordant findings in some reports were related subsequently to the isotope or culture medium used [11–13]. A predominant role for T-lymphocytes in the patho-

Abbreviations used: AICARibotide, 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate; BQR, brequinar; DHFR, dihydrofolate reductase; FCS, foetal calf serum; LFM, leflunomide; MTX, methotrexate; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; PHA, phytohaemagglutinin; PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate.

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Scheme 1 Diagram of the mechanisms associated with the inhibition of purine synthesis by MTX (inhibition of step 1) leading to the accumulation of PP-ribose-P in blasting human T-lymphocytes and the allosteric activation of pyrimidine synthesis (bold curved arrow)

The role of purine and pyrimidine nucleotides in RNA, DNA, nucleotide-sugar and lipid synthesis in blasting T-lymphocytes is indicated (right panel), contrasting with resting human T-lymphocytes (left panel), which survive by salvaging hypoxanthine. The points at which the different radiolabelled substrates are incorporated after the up-regulation of these two synthetic pathways by mitogen stimulation are highlighted. The other enzymes putatively targeted by MTX, as well as those inhibited by LFM, BQR and the glutamine antagonist azaserine (AZA), are also shown. Step 1, identified in this study as being the target enzyme of MTX, is emphasized. The importance of PP-ribose-P as the substrate for the first enzyme of purine synthesis *de novo* and hypoxanthine salvage, as well as being an allosteric activator of pyrimidine biosynthesis [35], is evident. (Note that only substrates for enzymes potentially targeted by MTX, LFM, BQR and AZA are shown. Minor pathways in resting lymphocytes are indicated by dotted arrows.) Abbreviations: DHOA, dihydro-orotic acid; GAR, glycineamide ribotide; FGAR, *N*-formyl glycineamide ribotide; OA, orotic acid; PPRP, 5-phosphoribosyl-1-pyrophosphate; THF, *N*¹⁰-methyltetrahydrofolate; THFM, *N*^{5,10}-methylene tetrahydrofolate.

genesis of rheumatoid arthritis has been proposed [11,12,15,22] but the beneficial action of MTX through a primary effect on T-cell function is disputed [8,9,14,19,20,23,24]. A widely accepted mechanism of action of low-dose MTX in rheumatoid arthritis is based on the hypothesis that MTX suppresses inflammation by mediating the release of adenosine [8,20,23,24]. Initially this was considered secondary to the inhibition by MTX of the folate-dependent enzyme AICARibotide transformylase (Scheme 1), leading to the intracellular accumulation of AICARibotide, as demonstrated with a mouse model *in vivo* of inflammation [20]. Subsequent studies implicated adenine nucleotides, released as a result of cellular injury or necrosis, as being the source of the extracellular adenosine, in which AICARibotide accumulation might be involved indirectly [24]. More recently it has been proposed that, in addition to adenosine release, the potent immunosuppressive properties of low-dose MTX relate to the

induction of apoptosis of activated T-cells in the S/G₂ phase of the cell cycle [26]. Such selective susceptibility of activated T-cells to MTX in rheumatoid arthritis patients could result in clonal deletion at the time of drug administration [26].

Our interest in MTX was stimulated by our studies in the T-cell immunodeficiency disorder purine nucleoside phosphorylase deficiency, and the neurological disorder of purine salvage, hypoxanthine-guanine phosphoribosyltransferase deficiency [27–29], in which AICARibotide diphosphate and triphosphate and the corresponding nucleosides and bases accumulate [29]. Our recent research on human T-lymphocytes [30,31] included the immunoregulatory drug leflunomide (LFM), which showed an efficacy similar to that of MTX in clinical trials [32]. This study demonstrated conclusively that the metabolic basis for the cytostatic action of LFM related to the inhibition of pyrimidine synthesis *de novo* [31] (Scheme 1). Importantly, LFM restricted

purine biosynthesis concomitantly [31]. Our earlier studies *in vitro* with other analogues also highlighted the many differences in their effects on ribonucleotide pools in primary human T-cells compared with malignant lymphoid cells, or human and rodent cells [30,33,34]. Although considerable attention has been paid to the changes in concentrations of the ribonucleotides ATP, GTP, UTP and CTP induced by MTX in malignant cells [3,4,6], surprisingly, no such studies have been performed in T-cells from healthy humans.

In the present study we investigated the effect of MTX with the same model *in vitro* as that employed for our studies on LFM [31]. The measurement of changes induced by MTX in ribonucleotide pools in human T-cells responding to mitogen, coupled with in-line radiodetection, has also avoided the complications identified in studies employing tritiated precursors [11,12,15]. All cultures were performed in medium that (1) contained heat-inactivated dialysed foetal calf serum (FCS) to circumvent any effects of hypoxanthine and uridine on nucleotide pools or purine-metabolizing enzymes in the medium and (2) was supplemented daily with glutamine to avoid the possibility of spurious results owing to glutamine exhaustion by MTX [4].

Our combined results show that MTX, like LFM, exerts an antiproliferative effect in mitogen-stimulated primary human T-lymphocytes cultured under identical conditions *in vitro* [31]. They also provide the first evidence that this relates to inhibition of purine biosynthesis *de novo* in this primary human T-lymphocyte model, not at the ninth step leading to AICARibotide accumulation but rather at the first committed step catalysed by amidophosphoribosyltransferase.

MATERIALS AND METHODS

Materials

Phytohaemagglutinin (PHA), trichloroacetic acid and all chemicals and standards for HPLC (analytical reagent or Aristar grade), RPMI without HCO_3^- , MTX, [$8\text{-}^{14}\text{C}$]hypoxanthine (1.07 mM; 49.6 Ci/mol), [$2\text{-}^{14}\text{C}$]uridine (0.89 mM; 56 Ci/mol) were from Sigma Chemical Co. (Poole, Dorset, U.K.). [$\text{U-}^{14}\text{C}$]Glycine (0.47 mM; 106 Ci/mol) was from ICN Biochemicals Ltd. (Thame, Oxon., U.K.) and $\text{H}^{14}\text{CO}_3^-$ (54 mCi/mmol) was from Amersham (Little Chalfont, Bucks., U.K.). RPMI-1640, Hanks balanced salt solution, heat-inactivated FCS, penicillin (10000 i.u./ml)/streptomycin (10 mg/ml) and 24-well plates were from Gibco (Paisley, Scotland). The mouse monoclonal anti-CD16 antibodies were from Coulter Electronics, goat anti-mouse antibody-coated Dynabeads were from Dynal Ltd. (Bromborough, Merseyside, U.K.), mouse monoclonal antiglycophorin A antibodies were from Dako Ltd. (High Wycombe, Bucks., U.K.) and Ficoll-Paque was from Pharmacia Biotech (St. Albans, Herts., U.K.). Culture supernatant was generated from a hybridoma secreting antibody specific to HLA-DR (clone L243; mouse IgG2a).

Preparation of PBMC

Heparinized blood from volunteers, or buffy coat from the North London Blood Transfusion Service, was diluted in RPMI-1640 and centrifuged over a Ficoll-Paque gradient. The PBMC were harvested, washed and resuspended in full medium [RPMI supplemented with 10% (v/v) heat-inactivated dialysed FCS, penicillin and streptomycin].

T-lymphocyte separation

T-lymphocytes were obtained from PBMC by monocyte, B-lymphocyte, NK-cell and reticulocyte depletion as follows. Cell

pellets were incubated with antibodies [25 μl of anti-HLA-DR, 1–2 μg of anti-glycophorin A and 0.2–0.5 μg of anti-CD16 per 10^6 target cells] at 4 °C for 30 min, then washed and incubated with Dynabeads (4:1 beads to target cells). The coated cells were depleted with a magnetic particle concentrator and the purified T-lymphocytes were washed and resuspended in 10 ml of full medium. The purity of the preparation was more than 96% T-cells.

T-lymphocyte studies

Experiments were performed with cell populations comprising 97% pure T-cells plus 3% PBMC acting as antigen-presenting cells. T-cells were preincubated in 0.9 ml of full medium [(1–1.3) $\times 10^6$ cells per well in a 24-well plate] either alone (control group) or in medium containing MTX at the concentrations indicated. After 30 min, 0.1 ml of 50 $\mu\text{g}/\text{ml}$ PHA was added and the cells were cultured at 37 °C in a humidified 5% CO_2 incubator for up to 72 h. Control non-stimulated cells were incubated for 72 h without PHA. The number of viable cells was always more than 85%. Because of the reported glutamine deprivation induced by MTX in cultured lymphoblasts [4], 10 μl of glutamine (200 mM) was added every 24 h to each well. At 24 h intervals, samples were taken for cell counting, cell-cycle analysis, evaluation of nucleotide concentrations and radiotracer studies.

Inhibitor concentrations

These were tested first in pilot studies performed with increasing concentrations of MTX from 20 nM to 20 μM , concentrations within the range attainable clinically [8,9,14].

Extraction and assay of ribonucleotides, nucleosides and bases

Reactions were stopped by centrifugation at 1000 g for 1 min; T-cells were washed once with Hanks balanced salt solution, centrifuged at 1000 g and the cell pellet was disrupted with 200 μl of 10% (w/v) trichloroacetic acid. Medium was also deproteinized with 25 μl of 40% (w/v) trichloroacetic acid. Both extracts were centrifuged for 1 min at 12000 g and trichloroacetic acid was removed by back-extraction with water-saturated diethyl ether to a pH of 5. Extracts were frozen at –20 °C if not analysed immediately by HPLC. Cell pellets were dissolved in 0.1 M NaOH and counted in a scintillation counter. Protein in the pellet was estimated by the Lowry method [33].

Short-term pulse-labelling studies

These studies involved 2 h incubations with the radiolabelled substrates listed below. At 24 h intervals for 72 h 10^6 T-cells from the above incubations, with or without inhibitors, were transferred into sterile 1.5 ml Eppendorf tubes and centrifuged at 1000 g for 1 min. Supernatant was discarded and 100 μl of fresh medium plus inhibitors was added with the appropriate radiolabelled substrate ([^{14}C]glycine at a final concentration of 90 μM , or [^{14}C]hypoxanthine or [^{14}C]uridine each at a final concentration of 50 μM) and incubated at 37 °C for 2 h. For $\text{H}^{14}\text{CO}_3^-$ the final concentration was 1.1 mM, the cells being incubated in tightly capped vials in HCO_3^- -deficient RPMI. Reactions were stopped by centrifugation at 1000 g for 1 min and the cell pellet and medium were extracted as above.

HPLC analyses

A Waters Trimodular system incorporating in-line photodiode array and radiodetection was used to quantify nucleotides as

described [30,31]. In brief, 100–175 μl of T-cell extract was injected on a Phenomenex Hypersil 5 μm (particle size) NH2-2 column (250 mm $\times$ 3.2 mm) at a flow rate of 0.5 ml/min with a linear phosphate buffer gradient: buffer A, 5 mM KH_2PO_4 ; buffer B, 0.5 M KH_2PO_4 /1.0 M KCl, initial pH 2.65 and 3.5 respectively. Nucleotides were quantified from their characteristic UV spectra and retention times were compared with authentic standards [30]. The rate and route of incorporation of radiolabel into nucleotide pools was followed by in-line radiodetection (Reeve Analytical, Glasgow, U.K.).

Statistical analysis

Statistical analyses were performed for ribonucleotide pool changes in response to PHA in the presence of MTX compared with T-lymphocytes stimulated with PHA alone. Data were analysed with a one-way analysis of variance, followed by the Student–Neuman–Keuls test to indicate individual differences.

Cell count, cell cycle, apoptosis and morphology

At 24 h intervals, individual wells seeded with 10^6 cells were harvested and centrifuged at 1000 g for 1.5 min, resuspended in 100 μl of RPMI containing 100 mg/ml *N*-acetylgalactosamine, incubated at 37 °C for 20 min, mixed gently and counted. Cell recovery and viability were assessed by the exclusion of Trypan Blue (Sigma). There was no significant decrease in cell number in either the stimulated or unstimulated cultures over the 72 h period. To assess cell cycle and apoptosis, 25 μl of the same cell suspension was placed in a 5 ml round-bottomed FACS tube at the same intervals, mixed with 1 ml of the lysis buffer (0.1 % sodium citrate/Triton X-100) containing 100 units/ml RNase A (Sigma), stained with propidium iodide (50 $\mu\text{g}/\text{ml}$) and stored in the dark at 4 °C for at least 30 min before FACS analysis. A total of 20000 events were acquired with a FACScan (Beckton Dickinson, Oxford, U.K.) for each sample. Histograms of propidium iodide fluorescence were generated with the use of Lysis2 software (Beckton Dickinson). Gates were set, by using the zero-time sample, to differentiate between G_0/G_1 (left-hand peak), S-phase (intermediate) and G_2/M (right-hand peak). Apoptotic cells appeared to the left of the G_0/G_1 peak. The remainder of the cell suspension was diluted with 100 μl of RPMI for the Cytospin analysis (50 rev./min for 10 min, four drops of cell suspension being used for each slide). Slides were dried, stained automatically with Grünwald–Giemsa stain, fixed under coverslips with DPX and examined by light microscopy.

RESULTS

Inhibitor concentrations

The pilot studies performed with MTX at concentrations ranging from 0.02 to 20 μM showed that MTX restricted the normal PHA-induced expansion of ATP over 72 h to concentrations of non-stimulated T-cells (Figure 1). All further experiments were performed with MTX at concentrations ranging from 0.1 to 2 μM .

Effect of MTX on viability and cell cycle in PHA-stimulated human T-lymphocytes

Cell counts confirmed that MTX inhibited the normal expansion of PHA-stimulated T-cells but was not cytotoxic. Cell numbers in non-stimulated cultures were not affected by MTX at any concentration (results not shown).

The results of cell cycle analysis, undertaken in parallel, using

MTX at 0.2 μM (Figure 2) and 2 μM (results not shown), demonstrated that the cells were arrested predominantly at the G_1 phase of the cycle. There was no difference in apoptosis between the T-cells stimulated with PHA alone (Figures 2A–2D) or preincubated with MTX before PHA stimulation (Figures 2E–2G) at any time point over 72 h. Morphological studies also excluded the possibility that necrosis occurred (results not shown).

Protein content of MTX-treated T-lymphocytes

The increase in protein content of T-cells stimulated with PHA alone over 72 h evident at the four time points measured (Figure 3C), contrasted with the relative stasis throughout in resting T-cells incubated for 72 h without PHA or inhibitors. The protein content in the T-cells treated with MTX (2–0.1 μM) showed a similar stasis at 24 h but increased slightly over the next 24 h of study. These results confirm the cell-cycle and morphology studies, demonstrating the absence of any cell death due to apoptosis or necrosis.

Effect of MTX on ribonucleotide pool expansion in human T-lymphocytes

Note that ribonucleotide pool concentrations (pmol/ 10^6 cells, y-axis) depicted in the histograms in Figures 3(A), 3(B) and 3(D)–3(F) are expressed on an initial cell basis (left histogram) to highlight their exponential expansion in T-cells stimulated with PHA alone and extracted at 24, 48 and 72 h thereafter. The greater expansion of the pyrimidine pools compared with the purine pools over 72 h in T-cells stimulated with PHA alone (Figures 3D–3F) was identical with that noted previously [30,31]. This contrasted with the relative stasis of the purine pools and the decrease in the pyrimidine pools in resting T-cells cultured for the same period, and the aberrations induced by MTX at 2.0, 1.0, 0.2 and 0.1 μM . The slight decrease in ATP and GTP concentrations in control cells in the first 24 h is consistent with their utilization for RNA and protein synthesis.

Restriction of normal PHA-induced purine nucleotide pool expansion in T-cells cultured with MTX

In contrast with the cells incubated with PHA alone, in T-cells preincubated and cultured throughout with MTX, the ATP and GTP concentrations remained at approximately those in freshly isolated cells over 72 h at all MTX concentrations (Figures 3A and 3B). The stasis in mean ATP and GTP concentrations induced by MTX compared with T-cells stimulated with PHA alone was highly significant at 72 h ($P \leq 0.001$) at all MTX concentrations. Differences were also significant at 48 h ($P \leq 0.001$ for ATP and $P \leq 0.03$ for GTP, at all MTX concentrations). The ratio of triphosphates to diphosphates, high in all instances, with little accumulation of monophosphates, confirmed the high-energy state of the cells (Figures 3A and 3B), as noted previously [30,31].

Enhanced pyrimidine nucleotide pool expansion in T-cells cultured with MTX compared with controls

Despite the above stasis in purine ribonucleotide pool expansion induced by MTX, and in contrast with our previous inhibitor studies [30,31], the PHA-induced expansion of UTP pools was not restricted correspondingly (Figure 3D). Expansion from 48 to 72 h was in fact generally greater than in the control, the increments being highly significant compared with T-cells stimu-

Figure 1 Effect of a range of MTX concentrations on ATP content of human T-lymphocytes

ATP concentrations (pmol/ 10^6 cells) in extracts of freshly isolated human T-lymphocytes measured at zero time and at 24, 48 and 72 h after either stimulation with PHA alone or preincubated with MTX before PHA stimulation at concentrations ranging from 0.02 to 20 μ M. Results are means for at least two separate experiments performed in duplicate.

lated with PHA alone at 48 h especially at 0.2 μ M ($P = 0.007$). Mean UTP concentrations at 48 h compared with PHA alone were also significantly higher at 2 μ M ($P \leq 0.01$) and 0.1 μ M

($P \leq 0.05$). However, at 72 h these differences were significant only at 0.2 μ M ($P \leq 0.05$). The disproportionate expansion of UTP pools induced by MTX could result from the accumulation of 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P), an allosteric activator of pyrimidine biosynthesis [34], owing to glutamine exhaustion in the medium [4]. This possibility was excluded because glutamine was added at 24 h intervals to all cultures.

Pulse-chase studies with ^{14} C-labelled precursors of synthesis *de novo*

^{14} C]Glycine incorporation into purine pools

Pulse-labelling studies with ^{14} C]glycine [incorporated into purine pools only (Scheme 1)] showed the expected exponential increment in T-cells stimulated with PHA alone from 24 to 72 h (Figure 4A). Although radiolabel incorporation into ATP and GTP in the presence of MTX was similar to that of the control at 24 h, incorporation declined sharply thereafter to 10% of the control at 72 h (Figure 4A), with no increment in ^{14} C accumulation in any intermediate of the pathway of purine synthesis *de novo*. Notably, there was no incorporation of radiolabel into peaks with retention times of AICARibotide, or (as noted previously with azaserine) *N*-formylglycinamide ribotide (Scheme 1) or its diphosphate and triphosphate [30]. These results confirmed the inhibition of purine synthesis by MTX but indicated that MTX induced a steadily increasing block from 24 to 72 h at or before the point of glycine incorporation in the pathway of purine synthesis *de novo* (Scheme 1, step 2).

Figure 2 Effects of MTX on cell cycling

Propidium iodide staining was performed at 24 h intervals in primary human T-cells cultured for 72 h. T-cells were studied before (A) and after stimulation with PHA alone (B–D) or were preincubated for 30 min with 0.2 μ M MTX before stimulation with PHA (E–G). Percentages shown indicate the proportions of cells in S-phase at the following time points: (A), 0 h; (B, E), 24 h; (C, F), 48 h; (D, G), 72 h. Each histogram consists of 20 000 events. The histograms are representative of identical results obtained with 2 μ M MTX.

Figure 3 MTX restricts purine but stimulates pyrimidine pool expansion in T-lymphocytes stimulated with PHA

Histograms depicting mean ribonucleotide concentrations (pmol/ 10^6 cells) and protein concentrations ($\mu\text{g}/10^6$ cells) in freshly isolated T-lymphocytes ($n = 8$) or after stimulation with PHA at 24, 48 and 72 h thereafter (+PHA), compared with concentrations in extracts of T-cells incubated without PHA (NS) over 72 h ($n = 5$) or T-cells preincubated with MTX at 2.0, 1.0, 0.2 and 0.1 μM ($n = 6$) before stimulation with PHA. Results are shown for purine nucleotides (A, B), protein (C), and pyrimidine nucleotides (D–F). The high ratio of triphosphates to diphosphates illustrates the excellent energy state of the cells throughout the 72 h. The S.E.M. in all instances was no more than 10% of the means presented. UDPG, UDP-glucose.

Inhibition of ATP and GTP synthesis *de novo* by MTX, demonstrated with $\text{H}^{14}\text{CO}_3^-$

In the cells incubated with MTX the results of the pulse-labelling studies with $\text{H}^{14}\text{CO}_3^-$, an early precursor in the pathways of both purine and pyrimidine synthesis *de novo* (Scheme 1), were similar to those noted with glycine. The incorporation of radiolabel into ATP and GTP (Figure 4B) was similar to that in the control at 24 h but declined thereafter over 72 h. As with glycine, there was no corresponding increase in any other peak in the chromatogram. In particular, no accumulation of radiolabel was noted in any peak with a retention time corresponding to AICARibotide, or its diphosphate or triphosphate, confirming the inhibition of purine synthesis at a point well before step 9 (Scheme 1). The UV traces confirmed these results: AICARibotide nucleotides have distinctive UV spectra and are therefore readily recognizable [30]. (The limit of detection for this method is 0.01 pmol/ 10^6 cells.)

Accelerated incorporation of $\text{H}^{14}\text{CO}_3^-$ into pyrimidine pools of MTX-treated cells

In contrast with the above restricted incorporation into purine pools, the incorporation of $\text{H}^{14}\text{CO}_3^-$ into UTP pools by the MTX-treated T-cells was comparable to, or even greater than, controls over 72 h (Figure 4C), although these differences were not statistically significant. However, such accelerated radiolabel

incorporation relative to controls confirmed the up-regulation of UTP synthesis by MTX that was evident from the nucleotide pools, despite the restriction of purine synthesis (Figures 3A and 3B).

A possible explanation for these combined results would be the up-regulation of pyrimidine biosynthesis by PP-ribose-P (an allosteric activator of the pathway [34]) after the inhibition by MTX of amidophosphoribosyltransferase [step 1 of purine biosynthesis (Scheme 1)]. Inhibition at this step would explain the lack of accumulation of radiolabel from $[\text{H}^{14}\text{C}]$ glycine or $\text{H}^{14}\text{CO}_3^-$ in any intermediate, despite the MTX-induced decline in incorporation into ATP and GTP (Figures 4A and 4B). To confirm this possibility, studies with $[\text{H}^{14}\text{C}]$ hypoxanthine were undertaken as a measure of PP-ribose-P availability [35].

$[\text{H}^{14}\text{C}]$ Hypoxanthine pulse-labelling studies confirm the up-regulation of purine salvage by MTX

Our contention [30,31] that resting T-cells 'tick over' (Scheme 1) through sustaining their ATP by hypoxanthine salvage was supported by the incorporation of $[\text{H}^{14}\text{C}]$ hypoxanthine mainly into ATP (Figure 5A). Incorporation into GTP as well as ATP increased linearly after stimulation with PHA from 24 h onwards, the ATP-to-GTP ratio being approx. 7:1 [30,31]. In contrast, in the MTX-treated T-cells, hypoxanthine salvage was greater than that in the T-cells stimulated with PHA alone at the three MTX concentrations and time points tested (Figure 5A). Incorporation

Figure 4 MTX inhibits *de novo* purine synthesis but stimulates *de novo* pyrimidine synthesis in T-lymphocytes stimulated with PHA

(A) The incorporation of [^{14} C]glycine into ATP (grey) and GTP (hatching) ribonucleotide pools of T-lymphocytes ($n = 6$) pulse-labelled for 2 h after stimulation with PHA at the time points indicated either alone or after preincubation with MTX at 2.0, 1.0 or 0.2 μ M; (B) the incorporation of $H^{14}CO_3^-$ into the UTP (vertical hatching) or CTP (black) pools of T-lymphocytes ($n = 4$) stimulated with PHA alone and pulse-labelled with $H^{14}CO_3^-$ for 2 h after incubation for the times indicated or preincubated with MTX at 2.0, 1.0, 0.2 or 0.1 μ M prior to stimulation with PHA. The S.E.M. was no more than 10% of the means presented.

into ATP and GTP was increased significantly compared with the control at 72 h by up to 50% ($P \leq 0.001$). These results contrasted with the relative stasis of salvage induced by either brequinar (BQR) or LFM in our earlier experiments in T-lymphocytes incubated with [^{14} C]hypoxanthine under identical conditions and [^{14} C]hypoxanthine concentrations [31]. Therefore the accelerated hypoxanthine salvage by the MTX-treated T-cells was not an artifact of the *in vitro* model used. The combined findings confirm an increased availability of PP-ribose-P in the MTX-treated cells, which is also consistent with the observed up-regulation of UTP synthesis [34]. These results, together with the steep decline in [^{14} C]glycine or $H^{14}CO_3^-$ incorporation into purine pools (Figures 4A and 4B) from 24 to 72 h confirm that the target enzyme of MTX in this *in vitro* model with pure human T-lymphocytes was not one of the folate-dependent steps (steps 3 and 9 in Scheme 1), but amidophosphoribosyltransferase, catalysing the first committed step of purine synthesis *de novo* (step 1 in Scheme 1).

Decreased incorporation of [^{14} C]uridine by T-lymphocytes incubated with MTX

As noted previously [31], uridine salvage into UTP, UDP-glucose and CTP was slight in freshly isolated T-cells, but increased from 24 to 72 h after stimulation with PHA (Figure 5B). In the MTX-treated T-cells, radiolabel incorporation into

all three pyrimidine nucleotides was virtually doubled at 24 h compared with PHA alone but declined thereafter (Figure 5B). This decline was attributed to the exaggerated up-regulation of pyrimidine synthesis *de novo* by MTX from 24 h onwards, thereby elevating UTP ribonucleotide concentrations disproportionately (Figure 3D), the latter exerting feedback control on uridine salvage. Again, results in identical pulse-labelling uridine salvage experiments with the two pyrimidine synthesis inhibitors BQR and LFM [31] illustrate the differing effects of MTX at 24, 48 and 72 h.

Ribonucleotide concentrations in T-cells after 2 h of pulse-labelling with [^{14} C]hypoxanthine

In the short-term (2 h) pulse-labelling experiments (Table 1), 50 μ M [^{14} C]hypoxanthine increased ATP and GTP pools slightly in the PHA-stimulated control T-cells and the unstimulated cells; however, these differences were not significant. More importantly, in the MTX-treated cells, despite the short-term nature of the incubations, the accelerated hypoxanthine salvage evident in Figure 5(A) not only restored ATP and GTP pools but also decreased the UTP pools in the MTX-treated cells concomitantly. These differences were significant at all three MTX concentrations: 2, 1 and 0.2 μ M. (For ATP, $P \leq 0.001$, $P < 0.001$, $P < 0.01$; for GTP, $P < 0.001$, $P < 0.01$, $P < 0.001$; for UTP, $P < 0.01$ in all instances). Such restoration was also

Figure 5 MTX stimulates hypoxanthine salvage, but restricts uridine salvage in T-lymphocytes stimulated with PHA

(A) The incorporation of [^{14}C]hypoxanthine into the ATP (grey) and GTP (hatching) pools of T-lymphocytes ($n = 4$) pulse-labelled for 2 h at zero time or at 24, 48 or 72 h after stimulation with PHA alone, or preincubated with MTX at the above concentrations before stimulation with PHA; (B) the incorporation of [^{14}C]uridine into UTP (vertical hatching), UDP-glucose (hatching) and CTP (black) of the same T-lymphocytes stimulated with PHA alone or preincubated with MTX before stimulation with PHA. The S.E.M. was no more than 10% of the means presented. For comparison with results from earlier experiments using *de novo* pyrimidine synthesis inhibitors, BQR and LFM, see Figures 5 (B) and 5(C) in [31]. Results with PHA alone [31] were virtually identical with those for the MTX experiments in (A) and (B).

evident in the 2 h pulse-labelling experiments with 50 μM [^{14}C]hypoxanthine in the T-cells cultured with MTX for 24 and 48 h (results not shown), confirming the excellent viability of the cells in the MTX-treated cultures throughout the 72 h period. These findings are in accord with the cell cycle studies and indicate that the decrease in purine pools was due to inhibition of purine synthesis *de novo*, not to poor viability or cell loss. The negation of the MTX-induced changes by hypoxanthine in these short-term incubations underlines the importance of using dialysed FCS in such *in vitro* models. The high concentration of hypoxanthine (and uridine) in undialysed FCS would mask the effects observed at all MTX concentrations used in such experiments *in vitro*.

DISCUSSION

These studies on primary human T-lymphocytes provide the first substantive evidence that the beneficial effects of a single low weekly dose of MTX relate at least in part to a cytostatic effect on purine biosynthesis and thereby on T-lymphocyte proliferation. MTX at concentrations attainable clinically (20 nM to 20 μM) induced stasis of blastogenesis at the G_1 phase of the cycle, but was cytostatic rather than cytotoxic to stimulated human T-cells. Neither apoptosis nor necrosis was noted and MTX showed no toxicity to non-proliferating T-cells. The results also demonstrate conclusively that the stasis of blastogenesis induced by MTX in T-lymphocytes relates not to inhibition of the two folate-dependent enzymes of purine biosynthesis (steps 3 and 9 in Scheme 1) but to the first enzyme of the pathway, amidophosphoribosyltransferase. The decreased incorporation of $\text{H}^{14}\text{CO}_3^-$ and [^{14}C]glycine into purine ribonucleotides, and the absence of radiolabel accumulation in any *de novo* intermediate, confirmed the inhibition of amidophosphoribosyltransferase by MTX. The effects of MTX in this human T-lymphocyte model thus differ from those in tumour cell lines in which the target enzymes implicated have been AICARibotide transformylase and thymidylate synthetase as well as DHFR [2–4,7,26]. They also contrast with the cytotoxic effect reported in activated human peripheral blood lymphocytes (PBL), in which MTX induces cell death through apoptosis [26], but concur with reports by some workers showing that MTX is not toxic to non-dividing PBL [4,26]. Although MTX clearly restricted purine synthesis and hence ATP and GTP pools to the concentrations of unstimulated T-cells, the noteworthy finding was that MTX actually stimulated pyrimidine biosynthesis. The increment in UTP pools as well as the incorporation of $\text{H}^{14}\text{CO}_3^-$ into UTP exceeded that of the PHA-stimulated control T-cells. Confirmation that the molecular mechanism underlying this up-regulation of pyrimidine biosynthesis by MTX was an increased availability of PP-ribose-P consequent on the inhibition of amidophosphoribosyltransferase [34] was obtained from the grossly accelerated salvage of [^{14}C]hypoxanthine. Importantly, utilization of PP-ribose-P by hypoxanthine normalized both the purine and UTP pools, despite the short-term (2 h) nature of these experiments. The latter observation might be equally important. For instance, long-term incubations in undialysed serum might mask the effects observed here *in vitro*.

Comparison of the present studies on human T-lymphocytes with reports by previous investigators on cell lines provides a logical explanation for the variation in findings reported, namely that the effect of MTX depends not only on the extent of up-

Table 1 Effect of short-term incubations with hypoxanthine on nucleotide concentrations in T-lymphocytes

Mean concentrations, after 2 h pulse-labelling experiments with 50 μM [^{14}C]hypoxanthine (Hx), of ATP, GTP and UTP (pmol/ 10^6 cells; means $\pm$ S.E.M., $n = 4$) in PHA-stimulated T-cells previously incubated for 72 h in medium containing dialysed heat-inactivated FCS in the absence or presence of MTX as indicated. Results are compared with mean ATP, GTP and UTP concentrations in identical cultures incubated for 72 h only in the absence or presence of MTX. These short-term pulse-labelling experiments were also performed in non-stimulated T-cells from the same donors, cultured for 72 h without PHA. * $P < 0.05$ compared with non-stimulated lymphocytes; † $P < 0.05$ compared with no MTX; ‡ $P < 0.05$ compared with no Hx.

Non-stimulated T-lymphocytes		PHA-stimulated T-lymphocytes		PHA-stimulated T-lymphocytes + MTX					
				2 μM MTX		1 μM MTX		0.2 μM MTX	
MTX	+ 50 μM Hx	MTX	+ 50 μM Hx	MTX	+ 50 μM Hx	MTX	+ 50 μM Hx	MTX	+ 50 μM Hx
ATP	669 $\pm$ 69	686 $\pm$ 73	1293 $\pm$ 117*	1385 $\pm$ 131*	630 $\pm$ 53†	1097 $\pm$ 47*‡	648 $\pm$ 38†	1161 $\pm$ 77*‡	642 $\pm$ 45†
GTP	140 $\pm$ 23	154 $\pm$ 34	317 $\pm$ 57*	330 $\pm$ 55*	187 $\pm$ 15	294 $\pm$ 10	197 $\pm$ 16	314 $\pm$ 34*	184 $\pm$ 10
UTP	103 $\pm$ 26	77 $\pm$ 15	264 $\pm$ 40*	303 $\pm$ 43*	311 $\pm$ 15*	217 $\pm$ 11*	336 $\pm$ 14*	219 $\pm$ 27*‡	311 $\pm$ 11*
									219 $\pm$ 19*

regulation of these *de novo* pathways in the particular cell type but also on the concentration of the drug used. For example, the accumulation of AICARibotide in MTX-treated breast cancer cells [2] clearly indicated blockage by MTX at AICARibotide transformylase, the ninth folate-dependent step (Scheme 1). In another study in malignant human T-lymphoblasts, MTX affected viability and growth at 0.2 μ M but not at 0.02 μ M; AICARibotide accumulated only at 0.2 μ M MTX [3,4]. Others argued from their model that folate depletion should lead to the inhibition of the first folate-dependent step catalysed by glycylamide ribotide transformylase, not AICARibotide transformylase [5], whereas studies in murine leukaemia L1210 cells showed a complete inhibition of purine synthesis by MTX at the first step (Scheme 1), catalysed by amidophosphoribosyltransferase [6].

Our human T-lymphocyte studies concur with the results from L1210 cells. The molecular mechanism underlying the inhibition of amidophosphoribosyltransferase by MTX in human T-lymphocytes is undoubtedly the formation of polyglutamated derivatives of folate or MTX, as demonstrated by Sant et al. [6]. Although the instability of the mammalian enzyme precluded characterization studies, inhibition of amidophosphoribosyltransferase by polyglutamated dihydrofolates or methotrexates was confirmed by using extracts of their L1210 cells [6]. The clear demonstration in the present study of the inhibition by MTX of step 1 of the purine pathway would explain why we found no evidence of AICARibotide, or of AICARiboside triphosphate, which might have been expected if AICARibotide had accumulated. Our methods regularly detect these compounds in cells and body fluids of patients with Lesch-Nyhan syndrome [29].

The normal incorporation of [^{14} C]glycine in our T-lymphocyte model at 24 h can also be explained by the initial rapid up-regulation of PP-ribose-P synthetase and purine synthesis [36] preceding the formation of polyglutamated derivatives. Subsequent accumulation would inhibit amidophosphoribosyltransferase progressively thereafter, as confirmed by the steady decline in radiolabel incorporation from 24 to 72 h. This sequence of events would be consistent with the sustained intracellular accumulation of polyglutamated methotrexates reported in cells of rheumatoid arthritis patients on low-dose MTX [37]. Such polyglutamated derivatives are not transported readily across membranes (reviewed in [7,8,26]) and are therefore retained intracellularly, thereby explaining the efficacy of a single weekly dose. The prolonged up-regulation of UTP synthesis over 72 h by MTX in these human T-lymphocytes contrasts with the effects reported for MTX in malignant cells in which the disproportionate increment in pyrimidine pools was maximal at 2 or 24 h, returning to normal by 8 and 48 h respectively [2–4]. Again, these differences must relate to the gross pre-existing up-regulation of purine synthetic enzymes in malignant lymphoblasts, thereby enabling them to overcome the MTX-induced block fairly rapidly [3,4,38]. In contrast, both pathways of synthesis *de novo* are essentially inactive in resting T-lymphocytes [30,31].

The co-ordinate up-regulation of pyrimidine biosynthesis by MTX in stimulated human T-lymphocytes found here contrasted with our earlier studies, in which the inhibition of one pathway by azaserine, LFM or BQR was accompanied by a parallel restriction in the expansion of the other [30,31]. This led us to suggest that the co-ordinate up-regulation of pyrimidine (or purine) synthesis reported by others must relate to the fact that the studies were performed in malignant cells in which both synthetic pathways are permanently up-regulated, the extent varying considerably between species as well as between cell types [3,38,39]. The lack of any such co-ordinate up-regulation in those previous studies can be explained only by the inhibition of

PP-ribose-P synthetase and hence PP-ribose-P synthesis: i.e. the step before the first committed step of purine synthesis *de novo* (Scheme 1).

Our present results on stimulated T-lymphocytes cultured with MTX also differ in several respects from other reports with normal human cell types. Some of the latter related the anti-inflammatory properties of MTX to adenosine released either directly consequent on the accumulation of AICARibotide [20] or indirectly from the accumulation of adenine nucleotides extracellularly after cell death by the action of cells expressing ecto-5'-nucleotidase [22,24]. The energy charge in our MTX-treated primary human T-lymphocytes was extremely high; AMP being virtually undetectable would exclude this possibility. An alternative or additional mechanism proposed, namely the MTX-induced apoptosis of mitogen-stimulated PBL, which could induce clonal deletion of activated T-cells in the S/G₂ phase of the cell cycle *in vivo* [26], is also excluded by our cell cycle studies. The complete reversal by hypoxanthine of the MTX-induced stasis of purine pools at 24, 48 or 72 h in the short-term pulse-labelling studies would also exclude the possibility that MTX induces DNA or membrane damage and apoptosis in these T-cells, or extracellular nucleotide accumulation or loss through necrosis.

Because studies by various workers have employed similar low MTX concentrations, these differences must relate to variations in experimental design, or the conditions used *in vitro*, as well as the cell type. Up-regulation of nucleotide biosynthesis in PBL is also much greater than in pure T-cells [31] and the apoptosis reported in the S/G₂ phase was noted in blasting PBL stimulated with PHA alone for 72 h before treatment with MTX [26]. Up-regulation of purine biosynthesis in PBL under such circumstances would resemble that of immortalized T-cells more closely, thus differing from our pure human T-lymphocytes, which were preincubated with MTX before stimulation. The lack of toxicity of MTX to resting T-lymphocytes or PBL demonstrated by us and by others [4,26] should also apply to non-dividing human cells such as neutrophils and monocytes that lack synthesis *de novo*. Because the transformation of monocytes to macrophages occurs without cell division [40], none of the above effects, including AICARibotide accumulation, would be expected.

The antiproliferative effect of MTX demonstrated here in stimulated human T-lymphocytes does not exclude a possible anti-inflammatory action due to the accumulation of AMP/adenosine by other cell types in situations of ischaemia or hypoxia *in vivo* [24]. However, other evidence suggests that our observations *in vitro* might be relevant *in vivo* in patients with rheumatoid arthritis. The effect of MTX in increasing PP-ribose-P availability in human T-lymphocytes could explain the toxic response to 5-fluorouracil in patients treated with MTX [9,10,41]. The salvage of 5-fluorouracil by orotic acid phosphoribosyltransferase (the first enzyme of the UMPS complex catalysing the last two steps of pyrimidine biosynthesis) is dependent on PP-ribose-P (Scheme 1). Indeed, the increased availability of PP-ribose-P induced by MTX forms the basis of the synergistic action of many anti-cancer combination therapies, including 5-fluorouracil [3,4,42]. Given the importance of pyrimidine nucleotides in membrane synthesis, structure and function [30,43,44], the disproportionate increase in UTP pools induced by MTX, while inducing stasis in purine pools, might also produce unbalanced growth. The latter would be deleterious to all dividing cells. It remains to be determined whether such an imbalance might contribute to the bizarre nodular changes noted in MTX-treated patients [9,10,45]. Our finding that hypoxanthine (even in the short term) reversed the concomitant restriction of purine pools and up-regulation of pyrimidine pools in human T-cells

suggests that hypoxanthine might be useful in overcoming side effects related to aberrant cellular nucleotide changes in rheumatoid arthritis. Approx. 30% of patients reportedly abandon treatment because of drug-related effects [25]. Additionally, any situation in which the hypoxanthine level is grossly elevated (e.g. hypoxia or ischaemia) might reverse the immunosuppressive effects of MTX in T-cells and underlie variable responses to therapy.

In summary, our studies show that MTX at the low concentrations used in rheumatoid arthritis would inhibit human T-lymphocyte proliferation and associated effector functions. However, neither of the two folate-dependent steps is the target enzyme. The molecular mechanisms underlying the cytostatic action of MTX in human T-lymphocytes relate to the inhibition of amidophosphoribosyltransferase, the enzyme catalysing the first committed step of purine synthesis *de novo*. Sustained inhibition after the intracellular accumulation of inhibitory polyglutamated derivatives of dihydrofolate or MTX could explain the efficacy of a single weekly dose [37]. Our findings do not exclude the possibility of the clonal deletion of previously activated T-cells by MTX *in vivo* [26] but such a complete inhibition of amidophosphoribosyltransferase would make AICARibotide accumulation impossible [20] except in such pre-activated T-cells. Different findings in other studies might relate to our use of primary human T-lymphocytes as opposed to malignant or preactivated cells, in which high basal metabolic rates would mask these effects. The results imply the involvement of low-dose MTX in the control of T-lymphocyte proliferation and proliferation-dependent processes in rheumatoid arthritis. Similar studies in T-lymphocytes of rheumatoid arthritis patients, and other cell types from healthy humans, are warranted.

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