Leflunomide Inhibits Pyrimidine de Novo Synthesis in Mitogen-stimulated T-lymphocytes from Healthy Humans

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Katarzyna Rückemann**, Lynette D. Fairbanks†, Elizabeth A. Carrey‡, Catherine M. Hawrylowicz§, David F. Richards¶, Bernhard Kirschbaum‖, and H. Anne Simmonds‡ ‡‡

From the **Purine Research Laboratory and the †Department of Allergy and Respiratory Medicine, United Medical and Dental Schools of Guy’s and St. Thomas’ Hospitals, London Bridge SE1 9RT, Great Britain, the ‡Department of Biochemistry and Molecular Biology, Royal Free Hospital School of Medicine, London NW3 2PF, Great Britain, and ‡‡Hoechst Marion Roussel, Frankfurt D-65926, Germany

The mode of action of Leflunomide, an immunomodulatory drug used in rheumatoid arthritis, is debated. This study, using [14C]-labeled de novo purine and pyrimidine synthesis precursors, proves conclusively that the prime target in proliferating human T-lymphocytes is pyrimidine biosynthesis at the level of dihydroorotic-acid dehydrogenase. Leflunomide (25 and 50 μM), like Brequinar (0.5 and 1 μM), a demonstrated dihydroorotic-acid dehydrogenase inhibitor, was cytostatic, not cytotoxic, with proliferation being halted in the G1 phase. Both drugs restricted the normal 4–8-fold mitogen-induced expansion of pyrimidine pools over 72 h to concentrations found in nonstimulated T-cells and [14C]bicarbonate incorporation into UTP, ATP, and GTP. Uridine (50 μM) restored expansion of all pools, but [14C]bicarbonate incorporation into ATP and GTP only, not UTP. [14C]Hypoxanthine salvage was also restricted, indicating that purine salvage pathways are compromised likewise by both inhibitors. [14C]Glycine studies confirmed that restriction of de novo purine synthesis occurred secondary to inhibition of proliferation since this was reversed by uridine rescue, except at 100 μM Leflunomide. 100 μM Leflunomide markedly depleted ATP and GTP pools also, which would have serious consequences for ATP-dependent enzymes essential to the immune response, thereby explaining non-pyrimidine-related effects reported for Leflunomide at 100 μM and above.

Leflunomide is a novel drug with both anti-inflammatory and immunoregulatory properties (1–3). Its mode of action is controversial. It is considered by some to resemble Brequinar (BQR), a known inhibitor of de novo pyrimidine synthesis at the level of dihydroorotic-acid dehydrogenase (DHODH) (Fig. 1A) (4–7). BQR is a potent antimetabolite used in solid tumors, but was withdrawn from clinical trial in organ transplantation because of toxic side effects (8–12). Leflunomide is converted rapidly in vivo to its active metabolite, A77 1726 (Fig. 1B), which has demonstrated efficacy in various animal models of autoimmune disease and also in preventing rejection after organ transplantation (2, 4, 13). A77 1726 is referred to as Leflunomide throughout this study and abbreviated as LFM. Trials of LFM in rheumatoid arthritis have shown good tolerance and statistically significant improvement in primary and secondary outcome measures (4, 14). Results of a multicenter Phase III clinical trial are awaited.

Putative molecular mechanisms of action of LFM in rheumatoid arthritis include inhibition of interleukin-2 production or receptor expression at either the level of gene transcription (2, 15–18) or via post-translational events and Th2-dependent B-cell functions secondary to this (1–3, 5, 13, 19, 20). Inhibition of various tyrosine kinases has been implicated from animal models (21–24), but the inhibitory concentrations of LFM (>100 μM) are much higher than the IC50 values for rat lymphocytes, 86 nm, and the 3.5 μM reported for mouse lymphocytes, or the 12.5 μM reported for human lymphocytes (5). Other potent effects appear to include both inhibition of adhesion and migration of inflammatory cells, enhancement of macrophage differentiation, and inhibition of cytokine action through down-regulation of receptor expression (1–3).

A considerable body of evidence now favors inhibition of de novo pyrimidine synthesis (3, 4–7, 13, 25, 26) by LFM at the level of DHODH (Fig. 1), the enzyme inhibited by BQR (9–11). This belief has been reinforced by studies demonstrating inhibition of pyrimidine synthesis by LFM with accumulation of dihydroorotic acid (DHOA) in human T-lymphoblastoid lines (7, 27) and inhibition of rat T-lymphocyte proliferation beyond the early S phase of the cell cycle, without causing cell death (5). In support of inhibition of de novo pyrimidine synthesis, both LFM and BQR (using either purified enzyme or lysed cell preparations in vitro) have been shown to be noncompetitive inhibitors of DHODH and to occupy the same site on the enzyme (10, 12, 25, 27–29). The fact that the antiproliferative effect of LFM in mouse lymphocytes, human lymphoblastoid cells, and peripheral blood mononuclear cells in vitro was reversed completely by uridine, but not guanosine, was considered evidence that the drug inhibited pyrimidine, but not purine, metabolism (6, 7). In vitro studies in human peripheral blood mononuclear cells have also confirmed LFM to be inhibitory at concentrations well within the IC50 range (6). However, supporters of the tyrosine kinase inhibition model have criticized these findings as being an in vitro phenomenon. Pyrimidine nucleotide pools were not reduced by LFM in vivo in splenic lymph node cells of MRL/MpJ-lpr/lpr mice, despite

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** Present address: Biochemistry Dept., Academic Medical School, Gdansk, Poland.

†† To whom correspondence should be addressed: Purine Research Lab., 5th Floor Thomas Guy House, UMDS Guy’s Hospital, London Bridge SE1 9RT, Great Britain. Tel.: 44-171-955-2438; Fax: 44-171-407-6689; E-mail: a.simmonds@umds.ac.uk.

§ The abbreviations used are: BQR, Brequinar; DHODH, dihydroorotic-acid dehydrogenase; DHOA, dihydroorotic acid; LFM, Leflunomide active metabolite A77 1726; PHA, phytohemagglutinin; HPLC, high performance liquid chromatography.
Amelioration of lymphoproliferative and autoimmune disease (24).

A possible explanation for the many conflicting reports relating to LFM could lie in the use of rodents that are not appropriate models for the study of purine or pyrimidine metabolism in human lymphocytes (30). The IC50 for rat DHODH with LFM is in the nanomolar range, whereas that in mouse or human lymphocytes is in the low micromolar range, as indicated above (29). A similar variation in potency between human and rodent DHODHs has been established for BQR (29).

The sizes of the ribonucleotide pools, as well as their routes of synthesis and salvage, also differ among human T-lymphocytes, peripheral blood mononuclear cells, and lymphoblastic cell lines (30–33). Chong and co-workers (21, 24) have used the argument that children with hereditary orotic aciduria present with anemia and not immunodeficiency to support their hypothesis.
hyponal immunosuppressive action of LFM. However, this could equally be explained by genetic heterogeneity (34). Severe affected children genetically deficient in the bifunctional enzyme UMP synthase, which catalyzes the last two steps of de novo pyrimidine synthesis (Fig. 1), do present with immunodeficiency as well as the more common form of presentation, refractory megaloblastic anemia and orotic aciduria (34). Moreover, the above contention is based on studies in rodents (15, 17, 21–24) that underline other important species differences, namely that human T-lymphocytes, even when stimulated, do not utilize orotic acid for pyrimidine nucleotide synthesis, whereas rodent lymphocytes and immortalized lymphoblastoid cell lines do (6, 7, 9, 30, 31). More important, although human erythrocytes cannot salvage uridine, they actively scavenge orotic acid, which they recycle and excrete as uridine for use by other tissues and cells such as T-lymphocytes (30).

In this study, we have addressed the above problems relating to in vitro studies and species differences, using model systems recently developed by us that demonstrated the particular importance of pyrimidine ribonucleotide availability to T-lymphocytes from healthy humans proliferating in response to phytohemagglutinin (PHA) stimulation (31, 35). We have used the same sensitive HPLC techniques coupled in-line radiodetection to follow changes in ribonucleotide pools in stimulated human T-lymphocytes incubated with LFM. The studies have included parallel experiments with BQR, a known pyrimidine biosynthesis inhibitor at the level of DHODH (9–11). The experiments presented have enabled us to demonstrate conclusively that LFM exerts its immunomodulatory effect by inhibiting the fourth enzyme of de novo pyrimidine synthesis in human T-lymphocytes.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—PHA, trichloroacetic acid, all chemicals and standards for HPLC (analar or Aristar grade), and RPMI 1640 medium without bicarbonate were purchased from Sigma (Poole, United Kingdom). [1-14C]Uracil (54 Ci/mmol) was from Amersham Life Science Ltd. (Little Chalfont, UK) [8-14C]Hypoxanthine (1.07 mM, 49.6 Ci/mol) and [2-14C]Uridine (0.89 mM, 56 Ci/mol) were purchased from Sigma. [U-14C]Glycine (0.47 mM, 106 Ci/mol) and [14C]Hypoxanthine (1.07 mM, 49.6 Ci/mol) and [2-14C]Uridine (0.89 mM, 56 Ci/mol) were purchased from Sigma. [U-14C]Glycine (0.47 mM, 106 Ci/mol) and [14C]Hypoxanthine (1.07 mM, 49.6 Ci/mol) were purchased from ICNBiothe purified T-lymphocytes were washed and resuspended in 10 ml of phosphate buffered saline, heat-inactivated fetal calf serum, penicillin (10,000 units/ml)/ streptomycin. Peripheral blood mononuclear cells isolated from buffy coats were stored overnight (12 h) at 4 °C.

**Cell Count, Cell Cycle, Apoptosis, and Morphology**—At 48-hour intervals, 1 × 10⁶ cells were harvested, incubated in 100 μl of RPMI 1640 medium containing 100 μg/ml N-acetylgalactosamine at 37 °C for 20 min, and counted. There was no significant decrease in cell number in any cultures over 72 h. 25 μl of this cell suspension were mixed with 1 ml of lysis buffer (0.1% sodium citrate and Triton X-100) and stained with propidium iodide (50 μg/ml) in the dark at 4 °C for >30 min prior to fluorescence-activated cell sorter analysis. The remainder of the cell suspension was diluted with 100 μl of RPMI 1640 medium for the CYTOSPIN analysis (50 rpm for 10 min).Slides were then stained with Grünwald-Giemsa stain, fixed with D.P.X., and examined by light microscopy.

**RESULTS**

**Inhibitor Concentrations**

Pilot studies established the optimal conditions and kinetics relating to the inhibitory potency of LFM and BQR on purine and pyrimidine ribonucleotide pool expansion in PHA-stimulated human T-cells (Fig. 2). Fig. 2A demonstrates that both BQR and LFM restricted the normal PHA-induced expansion of ATP pools over 72 h, with concentrations remaining at the level of freshly isolated T-cells. However, ATP pools were re-

T-lymphocyte Studies—Experiments were carried out using both extremely pure T-cells or pure T-cells supplemented with 2–3% peripheral blood mononuclear cells as a source of antigen-presenting cells. Cells were cultured in full medium at 1–1.3 × 10⁶ cells/well (24-well plate) in full medium alone (control group) or containing inhibitors to a final concentration of 10 μM each. After 24 h, the cells were incubated at 37 °C in a humidified CO₂ incubator for 0, 24, 48, or 72 h. Control nonstimulated cells were incubated for 72 h in the absence of PHA. After 72 h, the number of viable cells in the cultures was always >85%.

**Radioactive Tracer Studies**—At 24-hour intervals, cells were removed from the wells into sterile 1.5-ml Eppendorf tubes and spun at 1000 × g for 1 min. The supernatant was discarded, and 100 μl of fresh medium plus inhibitors were added, together with the appropriate radiolabeled substrate. [1-14C]Bicarbonate was used at a final concentration of 1.1 mM, with the cells being incubated in tightly capped vials in bicarbonate-deficient RPMI 1640 medium. [1-14C]Glycine was used at a final concentration of 90 μM, and [1-14C]hypoxanthine and [1-14C]uridine at a final concentration 50 μM each. After incubation at 37 °C for 2 h, reactions were stopped by centrifugation at 1000 × g for 1 min, and the cell pellet and supernatant medium were processed as described below.

**Uracil and [14C]Uridine pools**—Nucleotides and Bases—Medium was removed and deproteinized with 25 μl of 40% trichloroacetic acid. T-cells were washed once with Hanks’ balanced salt solution and centrifuged at 1000 × g, and the cell pellet was disrupted with 200 μl of 10% trichloroacetic acid. Both medium and cell extracts were then centrifuged for 1 min at 12,000 × g, and trichloroacetic acid in the supernatants was removed by back-extraction with water-saturated diethylether to pH 5. Extracts were then washed at –20 °C if not analyzed immediately by HPLC. The cell pellets were dissolved in 0.1 M NaOH and counted in a scintillation counter. Protein in the pellet was estimated by the method of Lowry et al. (45).

**HPLC Analyses**—A Waters Trimodular system incorporating in-line photodiode array and radiodetection was used for the separation and quantitation of ribonucleotides and uridine acid. Extracts and assay standards were desorbed (31). 100–175 μl of T-cell extract were injected onto a Phenomenex Hypersil 5-μm NH₂-2 column (250 × 3.2 mm) at a flow rate of 0.5 ml/min using a linear phosphate buffer gradient elution system: buffer A (5 mM KH₂PO₄) and buffer B (0.5 M KH₂PO₄ and 1.0 M KC), initial pH 2.65 and 3.5, respectively. Nucleotides were quantified from the characteristic UV absorption spectra, and the retention time was compared with authentic standards (31, 35, 36). The rate and relative incorporation of radioactivity into the different nucleotide pools were followed using an in-line radiodetector (Reeve Analytical, Glasgow, Scotland). Data collection and processing were performed using Waters Millennium software; results were calculated with a Lotus 1-2-3 spreadsheet. No deoxynucleotide triphosphates were detected in any experiments (35).

**Statistical Analysis**—Statistical analysis of ribonucleotide concentration changes in response to PHA in the presence of inhibitors compared with control lymphocytes alone was performed using Student’s t test available in the Lotus 1-2-3 spreadsheet.

The experiments presented have enabled us to demonstrate conclusively that LFM exerts its immunomodulatory effect by inhibiting the fourth enzyme of de novo pyrimidine synthesis in human T-lymphocytes.
Note that ribonucleotide pool concentrations (picomoles/10^6 cells, y axis) depicted in the histograms in Fig. 3 are expressed on an initial cell basis to highlight the exponential expansion following PHA and the aberrations induced by the two inhibitors. All results in Fig. 3 (A–D) refer to pure T-cells. Results in Fig. 3 (E–H) as well as Figs. 4 and 5 and Table I refer to T-cells + peripheral blood mononuclear cells.

**Ribonucleotide Pools in Nonstimulated T-lymphocytes Cultured for 72 h Remain Static—**ATP and GTP pools remained essentially unchanged in T-cells cultured without PHA (n = 12) from 24 h onwards (Fig. 3, A and B), as did protein concentrations (Table I), whereas pyrimidine pools decreased by up to 50% by 72 h (Fig. 3, C and D).

**Pure T-lymphocytes Show an Extremely Blunted Response to PHA, with Only Pyrimidine Pools Being Reduced by Inhibitors—**The requirement of T-cell proliferation for antigen-presenting cells (pure T-cells; n = 9) was evident from the lack of change in ATP and GTP (p = 0.8 and 0.76, respectively) (Fig. 3, A and B) or protein concentrations (days 0–3, p = 0.7) (Table I) over the 72 h following PHA stimulation. ATP and GTP pools were also relatively unchanged when incubated with BQR or LFM, except for LFM at 100 μM, which reduced both by up to 50%. Pyrimidine pools in controls following PHA stimulation (Fig. 3, C and D) showed a minor increment in UTP and CTP (p = 0.27 and 0.17, respectively). Results using either inhibitor were virtually identical to those in T-cells incubated without PHA (p = 0.54 and 0.9, respectively).

**Marked Reduction in Normal Pyrimidine Nucleotide Pool Responses to PHA in T-cells + Peripheral Blood Mononuclear Cells Cultured with Inhibitors—**Purine pools in control T-cells + peripheral blood mononuclear cells (n = 9) (Fig. 3, E and F) expanded up to 2-fold when stimulated by PHA. By contrast, ATP and GTP pools in cells cultured with BQR and LFM remained static over 72 h (ATP, p = 0.2 for BQR and p = 0.6 for LFM; and GTP, p = 0.07 and 0.29, respectively), except for LFM at 100 μM (n = 4), which actually reduced both ATP and GTP by >50% from 24 h onwards (Fig. 3, E and F). The ratio of triphosphates to diphosphates and monophosphates, high in all instances, indicated the satisfactory energy state of the cells.

The PHA-induced expansion of pyrimidine pools in the stimulated control T-cells (Fig. 3, G and H) was disproportionately greater than for pure pools, as noted earlier (31). BQR not only inhibited this expansion, but concentrations were actually reduced from 24 h onwards, with a 60% depletion being evident by 72 h. The reduction with LFM at 50 μM and below was less than that for BQR at either concentration, but LFM at 100 μM reduced pyrimidine nucleotides by up to 85% over 72 h. These results were highly significant (days 0–3: UTP, p = 0.0031 for BQR and 0.0036 for LFM; UDP-Glu, p = 0.019 and 0.016, respectively; and CTP, p = 0.01 and 0.022, respectively).

**Uridine Rescue Experiments—**Addition of 50 μM uridine to the cultures prior to preincubation of T-cells with either LFM or BQR at the concentrations indicated (Table I) restored proliferation in tandem with the mitogen-induced increment in pyrimidine and purine pools. Protein content, which had remained at the level of nonstimulated T-cells in the PHA-stimulated T-cells cultured with either BQR or LFM, increased to the same degree as for the uninhibited PHA-stimulated cells in the cultures preincubated with 50 μM uridine. However, 50 μM uridine was unable to reverse the inhibitory effects of 100 μM LFM, with all ribonucleotide concentrations remaining at the same low levels as in the cultures without uridine (Table I). These findings provide unequivocal evidence that the cytostatic
effect of LFM, as for BQR, relates to inhibition of de novo pyrimidine synthesis.

**Pulse-Chase Studies with [14C]Bicarbonate**

Inhibition of de Novo UTP and CTP Synthesis by LFM and BQR Demonstrated Using Radiolabeled Bicarbonate—Pulse-labeling studies using [14C]bicarbonate, an early precursor in both purine and pyrimidine de novo pathways (Fig. 1), confirmed little purine or pyrimidine synthesis by resting lymphocytes from healthy subjects (data not shown). [14C]Bicarbonate incorporation into ATP, GTP, UTP, and CTP increased maximum by 48 h in PHA-stimulated cells, being similar to the
results at 72 h (Fig. 4, top left panel). However, the changes induced by the inhibitors were apparent from 24 h onwards, in accord with their effect on ribonucleotide pool expansion (Fig. 3) and the cell cycle studies. Radiolabel incorporation into UTP and CTP was blocked completely by both LFM and BQR, accompanied by accumulation of radiolabel in a major peak with a retention time of ~10 min in this anion-exchange system. Incorporation of radiolabel into ATP and GTP was reduced likewise by LFM and BQR (Fig. 4, left panels), in parallel with the inhibitory effect of both drugs on proliferation and on pyrimidine and purine ribonucleotide pool expansion shown in Fig. 3.

Uridine Rescue Experiments Incorporating [14C]Bicarbonate Confirm Sustained Inhibition of de Novo UTP and CTP Synthesis by LFM and BQR—The [14C]bicarbonate pulse-chase studies (Fig. 4, right panels) included in the uridine rescue experiments reported in Table I proved conclusively that LFM, like BQR, exerts its cytostatic effect in mitogen-stimulated human T-lymphocytes by inhibiting pyrimidine biosynthesis. Although preincubation with uridine restored [14C]bicarbonate incorporation into ATP and GTP pools (Fig. 4, right panels), there was still no incorporation into UTP or CTP in the T-cells cultured with either BQR or LFM. This finding, coupled with the sustained accumulation of radiolabel in precursors with the same retention times as in the studies without uridine (~5.7 and 10 min), confirmed that de novo pyrimidine synthesis was still completely blocked, despite normal proliferation and restoration of all ribonucleotide pools (Table I).

Using a system developed by us to separate ornithine from orotic acid following an allopurinol load test (37), we found that the main radiolabeled peak accumulating at 10 min (Fig. 4) in these human T-cells preincubated with either LFM or BQR was not DHOA. In a series of experiments involving also the synthesis of [14C]carbamoyl aspartate from [14C]aspartate (Fig. 1), the main metabolite with a retention time of ~10 min was found to be the immediate precursor of DHOA, [14C]carbamoyl aspartate (38).2 The small peak on the front of [14C]carbamoyl aspartate (Fig. 4), eluting ~0.5 min before it, was [14C]DHOA. The unidentified [14C]-labeled peak with a retention time of 5.7, which increased in the T-cells incubated with LFM and BQR, was found to be [14C]carbamoyl phosphate.2

[14C]Glycine Incorporation into Purine Pools Is Also Reduced by LFM and BQR—The pulse-labeling studies with [14C]glycine (Fig. 5A) were important for two reasons. First, they confirmed the absence of active de novo purine synthesis in resting T-cells prior to stimulation or in nonstimulated T-cells cultured for 72 h. The increased incorporation into ATP and GTP following PHA stimulation that paralleled the expansion of these pools over 72 h was also evident. However, incorporation into both was reduced up to ~70% of the control by both LFM and BQR, with little incorporation at all at 100 μM LFM, the latter resembling nonstimulated T-cells cultured for 72 h. This inhibition of [14C]glycine incorporation into purine pools was likewise completely reversed by uridine, except at 100 μM LFM, indicating that this restriction was secondary to the inhibition of pyrimidine biosynthesis induced by both drugs (data not shown).

[14C]Hypoxanthine Pulse-labeling Studies Confirm That Purine Salvage Is Restricted Also by Both BQR and LFM in T-lymphocytes—Although de novo purine synthesis was not detectable in nonstimulated T-cells, purine salvage was active as indicated by significant [14C]hypoxanthine incorporation by freshly isolated T-cells, confirming that resting lymphocytes survived and sustain their ATP by salvage, with little incorporation into GTP (Fig. 5B). Incorporation into GTP as well as ATP increased linearly after PHA (Fig. 5B). In the cells cultured with inhibitors, purine salvage was reduced by up to 60% by BQR or LFM. At 100 μM LFM, incorporation was greatly reduced, the results again resembling those of the T-cells incubated without PHA over 72 h.

[14C]Uridine Incorporation by T-lymphocytes Incubated with Both BQR and LFM in T-lymphocytes—As noted previously (31), uridine salvage was much less active than for hypoxanthine in freshly isolated T-cells. Uridine salvage was also minimal in cells incubated without PHA for 72 h (Fig. 5C). However, as anticipated from the uridine rescue studies, radiolabel incorporation into UTP and CTP following PHA stimulation increased from 24 to 72 h (Fig. 5C), being only slightly restricted by BQR or LFM, again except at 100 μM LFM.

Effect of Hypoxanthine and Uridine on Nucleotide Pools in Pulse-labeling Studies—The nucleotide pools in the T-cells incubated with 50 μM hypoxanthine in these short-term pulse-labeling studies changed little in the inhibited cells compared

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with uninhibited controls (data not shown), in accord with the marked restriction in purine salvage evident from the radiolabeling studies. By contrast, 50 μM uridine did increase pyrimidine pools in these short-term experiments, but the concentrations were still well below those of the uninhibited cells at 72 h (data not shown). More important, neither uridine nor hypoxanthine induced any significant increment in nucleotide concentrations in the cells incubated with 100 μM LFM (data not shown). The latter finding is consistent with the minimal incorporation of [14C]uridine or hypoxanthine by these T-lymphocytes noted above (Fig. 5, B and C) at this high LFM concentration.

DISCUSSION

These studies in T-lymphocytes from healthy humans were designed to assist resolution of the debate relating to the mechanism of action of LFM. We did this by carrying out parallel studies using the de novo pyrimidine biosynthesis inhibitor BQR, known to exert its immunosuppressive action at the level of DHODH (10). The pulse-labeling studies using [14C]bicarbonate and HPLC coupled with in-line radiodetection enabled us to demonstrate conclusively that the principal effect of LFM on mitogen-induced T-cell proliferation is inhibition of de novo pyrimidine synthesis. At concentrations that were cytostatic to T-lymphocyte proliferation, both LFM and BQR suppressed the normal ability of pyrimidine pools to expand in response to PHA. This applied to both uridine and cytidine nucleotide pools, with ATP and GTP pools remaining static over 72 h as well. The [14C]glycine studies confirmed that restriction of de novo purine synthesis occurred secondary to inhibition of proliferation since this was reversed by uridine rescue, except at 100 μM LFM.

Effects on both pyrimidine and purine pools were evident by 24 h, consistent with our cell cycle analysis and an earlier report that LFM arrests human T-lymphocyte proliferation in response to PHA at the G1 phase of the cell cycle (7). The less pronounced effects of LFM on pyrimidine pools over a wider concentration range compared with BQR indicate a broader therapeutic window for LFM. This in turn may explain the lower incidence of side effects with LFM in vivo (3, 5, 14). The fact that protein concentrations in the inhibited cells remained at the level of nonstimulated T-cells, coupled with the absence of any other indices of cell death, indicates that both drugs were cytostatic, not cytotoxic, even at 100 μM LFM. Neither showed any toxicity to non-proliferating T-cells.

The direct correlation between the anti-proliferative effect of LFM and BQR in T-lymphocytes and the depletion of intracellular pyrimidine pools was confirmed in two ways: first, by the fact that 50 μM uridine completely restored both proliferation and expansion of purine and pyrimidine nucleotide pools as well as [14C]glycine incorporation into ATP and GTP; and second, by the [14C]bicarbonate pulse-chase studies showing that, despite restoration of all ribonucleotide pools by uridine, radio-label was incorporated into ATP and GTP only, not UTP or CTP. The surprising finding was that the principal de novo pyrimidine synthesis intermediate accumulating in the presence of either BQR or LFM was not DHOA, as would be anticipated from inhibition of DHODH, but carbamoyl aspartate. Only small amounts of DHOA accumulated in these T-lymphocytes. This can be explained by the fact that the equilibrium constant for the reversible reaction catalyzed by dihydroorotase (Fig. 1) favors the formation of carbamoyl aspartate at physiological pH (39, 40). The sustained accumulation of these de novo intermediates, despite full restoration of nucleotide pools by uridine, showed that the block was still complete. The combined results confirm that LFM, like BQR, is a potent inhibitor of de novo pyrimidine synthesis at the level of DHODH in human T-lymphocytes, as in other cell types (7, 27, 34).

The question is, how do the observed metabolic changes relate to the putative modes of action of LFM and its effectiveness in rheumatoid arthritis? Considering first the effect of LFM on T-lymphocyte nucleotide profiles following PHA stimulation, the reduced expansion of pyrimidine pools is in marked...
contrast to the manyfold expansion that is clearly essential to normal human T-lymphocyte proliferation (31). The disproportionate expansion of pyrimidine relative to purine pools in normal T-lymphocytes is related to their additional requirement of UTP and CTP for other growth-related activities, such as lipid and protein glycosylation and membrane biosynthesis (Fig. 1). Induction of UTP depletion is known to be a key mechanism by which hexose analogues exert their growth inhibitory effects in human cells (31, 41, 42). CDP-choline is a vital intermediate in phospholipid synthesis, particularly the acidic phospholipids, the inositol polyphosphates involved in signal transduction and translation (31, 43). The restriction of UTP, UDP-Glu, and CTP pools by LFM and BQR to the level of nonstimulated cells would severely restrict all the above growth-related activities. Expansion of UTP pools is equally important to provide UDP-sugars for the nucleotide dolichol phosphate-linked sugar intermediates involved in the glycosylation of adhesion molecules (41, 42). Studies with the purine
synthesis inhibitor mycophenolic acid (Fig. 1) have demonstrated that GTP, too, is essential for the incorporation of mannose and fucose into glycoproteins and dolichol-linked intermediates via the corresponding GDP-sugar precursors (41). The reduced availability of GTP and thus GDP-sugars, as for the UDP-sugars discussed above, would similarly influence cell-surface topography and lectin binding, thereby restricting mitogenic responses (41, 42). Thus LFM, like BQR and mycophenolic acid, could restrict the glycosylation of adhesion molecules also, thereby providing a metabolic basis for the anti-inflammatory as well as the immunosuppressive effects proposed for LFM (2, 3, 41).

The studies with 100 μM LFM are particularly important when related to the proposals of others regarding the putative mechanisms of action of LFM in rheumatoid arthritis (15, 17, 21–24). The severe reduction in ATP and GTP as well as pyrimidine ribonucleotide pools in T-cells cultured with 100 μM LFM, coupled with the inhibition of incorporation of radiolabeled hypoxanthine or uridine, indicates complete stasis of metabolism in these cells. The cell count and protein studies confirmed that this was not due to cell loss. These findings explain the lack of restoration of nucleotide pools (or proliferation) in the uridine rescue experiments at this high LFM concentration. The results are in direct contrast to those reported using murine CTLL-4 cell lines, which showed that 100 μM LFM had no effect on ATP and GTP pools, whereas co-culture with 50 μM uridine effected complete restoration of UTP and CTP pools and reversed the inhibition of proliferation (17). These differences are significant and highlight the many problems related to the use of immortalized lymphoblastoid cells as well as the considerable interspecies variations in purine and pyrimidine metabolic pathways (30, 31).

Our present studies showed that high concentrations of BQR also (from 5 to 50 μM) had an effect identical to that of 100 μM LFM on ATP concentrations. The results accord with studies in murine colon tumor lines showing that uridine could only reverse the effects of BQR at concentrations below 30 μM, other undefined mechanisms being considered responsible for the irreversible toxicity at higher concentrations (11). Clearly, the marked reduction in ATP concentrations demonstrated in this study could provide a likely explanation for the effects reported for both LFM and BQR on other immunological parameters at high concentrations. ATP is vital for the activity of many enzymes, especially those involved in signal transduction, such as tyrosine kinases. Inhibitors being developed in the field of cancer chemotherapy target specific tyrosine kinases by occupying the binding site for the phosphate-donating molecule, ATP (44). Thus, the severe ATP depletion noted here could provide a biochemical basis for the non-pyrimidine effects reported by others for LFM at 100 μM (or above) involving different tyrosine kinases (15, 17, 19, 21–24).

The present studies confirm that small non-dividing T-cells from healthy humans replenish sufficient ATP through hypoxanthine salvage to keep their metabolic pathways “ticking over.” Uridine salvage, or de novo synthesis of either purines or pyrimidines, is minimal. However, when stimulated by PHA in the presence of either LFM or BQR, the impairment in normal pyrimidine nucleotide pool expansion makes it impossible for these T-lymphocytes to proliferate. Inhibition of pyrimidine biosynthesis in turn restricts de novo purine synthesis. Purine salvage pathways are equally compromised, all of which are essential to enable T-lymphocytes to enter the G1/S phase and to complete the cell cycle (27, 29–35). The fact that uridine restores proliferation and expansion of all nucleotide pools, but [14C]bicarbonate incorporation into purine pools only, demonstrates conclusively that LFM at concentrations below 100 μM exerts its immunomodulatory effect by inhibiting de novo pyrimidine biosynthesis. However, LFM at 100 μM and above induces complete stasis of metabolism in these human T-lymphocytes, which could severely compromise ATP-dependent processes, such as protein phosphorylation by tyrosine kinases.

The combined results are consistent with the importance of ribonucleotide availability to mitogen-stimulated T-lymphocytes for DNA synthesis and blast transformation demonstrated previously using known inhibitors of de novo purine synthesis (31). Clearly, pyrimidine ribonucleotide availability is equally crucial for regulating the magnitude and duration of the T-cell immune response. More important, restriction of the cascade of nucleotide-related events that normally follow mitogenic stimulation could contribute to the anti-inflammatory as well as the immunoregulatory effects of LFM in rheumatoid arthritis. The inhibitory effects of LFM on pyrimidine pool expansion could, as in AIDS, involve all cells undergoing active cell division (31, 35) and might explain the side effects noted in some rheumatoid arthritis patients involving the hematopoietic system, skin, and gut (14). Consequently, studies similar to those reported in this paper are essential in T-lymphocytes of rheumatoid arthritis patients prior to and following treatment with LFM. The finding that although DHODH is the target enzyme for LFM, DHOA does not accumulate in quantity in human T-lymphocytes also needs to be investigated in patients treated with LFM to determine whether these in vitro findings translate to the in vivo situation.

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REFERENCES

1. Bartlett, R., Campion, G., Musilak, P., Schleyerbach, R., Zielinski, T., and Schorlemmer, H.-U. (1994) in Nonsteroidal Anti-Inflammatory Drugs: Mechanisms and Clinical Uses (Lewis, A. J., and Forst, D. E., eds) pp. 349–366, Marcel Dekker, Inc., New York.
2. Bartlett, R. R., Dimitrijevic, M., Mattar, T., Zielinski, T., Germann, T., Rude, E., Thoenes, G. H., Kuchel, C. C. A., Schorlemmer, H.-U., Bremmer, E., Finnegan, A., and Schleyerbach, R. (1991) Agents Actions 32, 10–21.
3. Silva, H. T., Jr., and Morris, R. E. (1997) Exp. Opin. Invest. Drugs 6, 51–64.
4. Silva, H. T., Jr., Guo, W., Shorthouse, R. A., Loiffer, M., and Morris, R. E. (1997) Transplant. Proc. 29, 1292–1293.
5. Cherwinski, H. M., McCarty, D., Schatzman, R., Devene, B., and Ransom, J. T. (1995) J. Pharmacol. Exp. Ther. 272, 460–468.
6. Cherwinski, H. M., Byars, N., Ballaron, G., Nakano, G., Young, J., and Ransom, J. (1995) Inflammation Res. 44, 317–322.
7. Cherwinski, H. M., Cohn, H., Cheng, P., Webster, D., Xu, Y., Caulfield, J., Young, J., Nakano, G., and Ransom, J. (1995) J. Pharmacol. Exp. Ther. 275, 1043–1049.
8. Makowska, L., Sher, L., and Cramer, D. (1993) Immunol. Rev. 136, 51–70.
9. Peters, G. J., Sharma, E., Laurensse, E., and Pinedo, H. M. (1987) Invest. New Drugs 5, 375–381.
10. Peters, G. J., Schwartzmann, G., Nadal, J. C., Laurensse, E., Groeningen, C. J. V., Vijdj, W. J. F. V. D., and Pinedo, H. M. (1990) Cancer Res. 50, 4644–4649.
11. Peters, G. J., Kraai, L., and Pinedo, H. M. (1992) Br. J. Cancer 65, 229–233.
12. Simon, P., Townsend, R., Livermore, J., Jones, E., and Jaffer, B. (1993) Transplant. Proc. 25, Suppl. 2, 77–80.
13. Nair, R. V., Cao, W., and Morris, R. E. (1995) Immunol. Lett. 47, 171–174.
14. Madenovic, V., Donijan, Z., Rozman, B., Jagic, I., Mihajlovic, D., Desarevic, J., Popove, M., Dimitrijevic, M., Zvokovic, M., and Campion, G. (1995) Arthritis. Rheum. 38, 1595–1603.
15. Chong, A. S.-F., Reazi, K., Gehr, H., Finnegan, A., Foster, P., Xu, X., and Agar, J. (1996) Transplantation (Baltimore) 61, 140–147.
16. Mattar, T., Kocher, K., Bartlett, R., Bremmer, E. G., and Finnegan, A. (1993) FEBS Lett. 334, 161–164.
17. Nizkovitch, D. A., Finnegan, A., Chang, A. S.-F., Williams, J. J., and Bremmer, E. G. (1994) Agents Actions 41, C279–C282.
18. Samelson, L., Davidson, W., Morse, H., and Klausner, R. (1986) Nature 324, 674–676.
19. Sjemanke, K., Chong, A. S.-F., Williams, J., Bremner, E., and Finnegan, A. (1996) Transplantation (Baltimore) 61, 635–642.
20. Zielinski, T., Herrmann, M., Mullner, S., Riedel, N., and Bartlett, R. R. (1994) J. Pharmacol. Exp. Ther. 269, 229–233.
21. Elder, R., Xu, X., Williams, J., Gong, H., Finnegan, A., and Chong, A. S.-F. (1997) J. Immunol. 158, 22–27.
22. Xu, X., Williams, J. J., Bremner, E. G., Finnegan, A., and Chang, A. S.-F. (1995) Agents Actions 41, 205–207.
23. Xu, X., Williams, J. J., Gong, H., Finnegan, A., and Chong, A. S.-F. (1996) Biochem. Pharmacol. 52, 527–534.
24. Xu, X., Blinder, L., Shen, J., Gong, H., Finnegan, A., Williams, J. J., and...
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Chong, A. S.-F. (1997) J. Immunol. 159, 167–174
25. Williamson, R., Yen, C., Robson, P., Curnock, A., Gedhert, S., Hambleton, A., Woodwar, K., Bruneau, J. M., Hambleton, P., Moss, D., Thomsen, T. A., Spinella-Jaegle, S., Morand, P., Courtin, O., Sautès, C., Westwood, R., Herend, T., Kuo, E. A., and Ruuth, E. (1995) J. Biol. Chem. 270, 22467–22472
26. Zielinski, T., Zeitter, D., Mullner, S., and Bartlett, R. R. (1995) Inflammation Res. 44, S207–S208
27. Cleaveland, E. S., Monks, A., Vaigro-Wolff, A., Zaharevitz, D. W., Paull, K., Arslan, K., Conney, D. A., and Ford, J. R. (1995) Biochem. Pharmacol. 49, 947–954
28. Greene, S., Watanabe, K., Braatz-Trulson, J., and Lou, L. (1995) Biochem. Pharmacol. 50, 861–867
29. Knecht, W., Bergjohann, U., Gonski, S., Kirschbaum, B., and Löffler, M. (1996) Eur. J. Biochem. 240, 292–301
30. Simmonds, H. A. (1995) Biochem. Soc. Trans. 23, 877–879
31. Fairbanks, L., Bofill, M., Rückemann, K., and Simmonds, H. A. (1995) J. Biol. Chem. 270, 29682–29689
32. Marijnen, Y. M. T., de Korte, D., Haverkort, W. A., den Breejen, E. J. S., van Gennip, A. H., and Roos, D. (1993) Biochem. Biophys. Acta 1102, 148–155
33. van den Berg, A. A., van Lenthe, H., Busch, S., de Korte, D., van Kuijlenburg, A. B. P., and van Gennip, A. H. (1994) Leukemia (Baltimore) 8, 1375–1378
34. Webster, D. R., Becroft, D. M. O., and Suttle, D. P. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) 7th Ed., pp. 1725–1768, McGraw-Hill Book Co., New York
35. Bofill, M., Fairbanks, L., Rückemann, K., Lipman, M., and Simmonds, H. A. (1995) J. Biol. Chem. 270, 29690–29697
36. Simmonds, H. A., Duley, J. A., and Davies, P. M. (1991) in Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual (Hommes, F., ed) pp. 397–424, Wiley-Liss, New York
37. Sebesta, I., Fairbanks, L. D., Davies, P. M., Simmonds, H. A., and Leonard, J. V. (1994) Clin. Chem. Acta 224, 45–54
38. Hemmens, B. P., and Carrey, E. A. (1994) Eur. J. Biochem. 225, 845–853
39. Christopherson, R. L., and Jones, M. E. (1979) J. Biol. Chem. 254, 12506–12512
40. Kemp, A. J., Lyons, S. D., and Christopherson, R. I. (1986) J. Biol. Chem. 261, 14891–14895
41. Eugui, E. M., Almquist, S. J., Muller, C. D., and Allison, A. C. (1991) Scand. J. Immunol. 33, 161–173
42. Sokolowski, J. A., and Sartorelli, A. C. (1987) Int. J. Cancer 39, 764–768
43. Sasvari-Székely, M., Spasokukotskaja, T., and Staub, M. (1993) Biochem. Biophys. Res. Commun. 194, 966–972
44. Baringa, M. (1997) Science 278, 1036–1039
45. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275