Importance of Ribonucleotide Availability to Proliferating T-lymphocytes from Healthy Humans

DISPROPORTIONATE EXPANSION OF PYRIMIDINE POOLS AND CONTRASTING EFFECTS OF DE NOVO SYNTHESIS INHIBITORS

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Sensitive high performance liquid chromatography techniques, which differentiate between purine and pyrimidine ribonucleoside and deoxyribonucleoside triphosphates, were used to quantify pools in phytohemagglutinin-stimulated T-lymphocytes (98% CD4+ and CD8+) from healthy volunteers. The importance of de novo synthesis and salvage was evaluated by incubating the cells with 14C-radiolabeled precursors (40 µCi), aza- serine (20 µM; a glutamine antagonist), and ribavirin (50 µM; an IMP dehydrogenase inhibitor). We confirmed that resting T-lymphocytes meet their metabolic requirements by salvage.

Noteworthy observations were as follows. First, nucleotide pool expansion over 72 h is disproportionate, with that for purines (ATP and GTP) being 2-fold compared with up to 8-fold for pyrimidine (NAD) or pyrimidine (UTP, UDP-Glc, and CTP) pools. This supports an additional role for the latter in membrane lipid biosynthesis, protein glycosylation, and strand break repair. Second, intact de novo pathways are essential for such expansion. Azaserine not only inhibited purine synthesis (confirmed by N-formylglycinamide polyphosphate accumulation), but also reduced expansion of pyrimidine and NAD pools by 70%. Ribavirin depleted GTP pools by 40% and reduced pyrimidine pool expansion by 40% at 72 h.

These findings underline the importance of pyrimidine ribonucleotide availability as well as GTP synthesis de novo to proliferating T-lymphocytes. They also demonstrate an absence of coordinate regulation between de novo purine and pyrimidine biosynthesis.

Triggering of resting T-lymphocytes under physiological conditions by antigenic peptides associated with the major histocompatibility complex is a multistep process. Stimulation leads to the induction of biochemical signals that are transmitted sequentially from the cell surface to the nucleus, resulting in gene transcription (1–3). Replication of activated T-cells requires additional signals mediated by other costimulatory receptors or accessory molecules (such as CD4, CD8, CD2, leukocyte function-associated antigen, CD28, and CD45) or binding of growth-promoting cytokines (such as interleukin-1,-6, and -2). Failure to produce these costimulatory signals or the associated biochemical responses could lead to unresponsiveness or cell death instead of replication (1, 2). The activation of T-cells in vivo is usually mimicked in vitro by the addition of mitogens such as phytohemagglutinin (PHA)§ or by activation with anti-CD3 or anti-T-cell receptor antibodies (1). PHA causes the transformation of resting lymphocytes into rapidly dividing lymphoblasts, with the synthesis of DNA beginning at 24 h and peaking around 48 h, preceded by an increase in the rate of synthesis of RNA and protein (4, 5).

The importance of intact purine pathways to the normal immune response was highlighted by the discovery of the two potentially fatal genetic immunodeficiencies (purine-nucleoside phosphorylase deficiency and adenosine deaminase deficiency) associated with severe T-lymphocyte or T- and B-lymphocyte depletion and impaired mitogen responses (6, 7). This led to the development of novel adenosine deaminase and purine-nucleoside phosphorylase inhibitors for the treatment of hematological malignancies as well as immunosuppressive agents (3, 6, 8). Inhibitors targeting de novo pyrimidine biosynthesis or GTP synthesis at the level of IMP dehydrogenase (Fig. 1), an enzyme associated with T-cell activation and malignancy (3), have likewise been the focus of strategies aimed at overcoming allograft rejection (3, 9).

In the majority of in vitro studies, evaluation of the events following PHA stimulation has been indirect, based on the incorporation of tritiated thymidine, uridine, or leucine (3–5, 7, 10, 11). Data documenting ribonucleotide pools in resting T-lymphocytes and the actual changes occurring over 72 h in stimulated T-lymphocytes (an integral component of the above cascade (Fig. 1) that enables an immunocompetent host to respond to mitogenic agents in vivo) are scant (10–12). A number of studies have compared ribonucleotide pools in resting T-lymphocytes and the actual changes occurring over 72 h in stimulated T-lymphocytes (an integral component of the above cascade (Fig. 1) that enables an immunocompetent host to respond to mitogenic agents in vivo) are scant (10–12). A number of studies have compared ribonucleotide pools in resting T-lymphocytes with pools in malignant or virally infected cells (reviewed in Refs. 13–18). Others have focused on lymphocyte deoxyribonucleotide pool changes in response to PHA. However, these have been measured usually by indirect assay, and although a 10-fold expansion has been reported following PHA stimulation in peripheral blood lymphocytes, concentrations are extremely low compared with ribonucleotide pools (19–21). Only recently have the concentrations of both pyrimidine and purine ribonucleotides been compared in resting and stimulated T-lymphocytes and equated with the incorporation of 14C-radiolabeled precursors (12).

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§The abbreviations used are: PHA, phytohemagglutinin; HPLC, high performance liquid chromatography.
nucleotide pools over 72 h following mitogen stimulation with the evaluation of the effect of de novo synthesis inhibitors of both pathways on the ability of ribonucleotide pools in T-lymphocytes to expand in response to PHA stimulation.

To address these points, we separated T-lymphocytes (CD4+ and CD8+) from healthy donors, stimulated them with PHA, and followed changes in ribonucleotide concentrations over 72 h using highly sensitive techniques (high performance liquid chromatography (HPLC) with in-line photodiode array and radiodetection) (22). The HPLC method used was originally developed by us to demonstrate the erythrocyte dATP accumulation associated with ATP depletion in inherited adenosine deaminase deficiency and the GTP depletion and dGTP accumulation in nucleoside-phosphorylase deficiency (23).

More important, the method separates purine ribonucleotides from pyrimidine ribonucleotides as well as from their corresponding deoxyribonucleotides and sugar derivatives. The method is in daily use as a diagnostic tool (22) and for monitoring the decrease in dATP in adenosine deaminase-deficient patients treated with polyethylene glycol adenosine deaminase (6).

The experiments included the use of radiolabeled substrates and T-lymphocytes that were incubated in the presence of azaserine or ribavirin, known inhibitors of specific steps of de novo purine synthesis (15, 18). The objective was to determine how stimulated T-cells switch on their de novo synthetic pathways to provide the additional ribonucleotide precursors necessary for (a) RNA and DNA synthesis and (b) the other processes associated with cell division: repletion of energy stores (ATP) and the formation of cofactors (NAD), second messenger precursors, and the purine and pyrimidine sugars for membrane lipid synthesis and glycosylation (Fig. 1). The relative roles of salvage of preformed purines and pyrimidines in these processes was also evaluated to enable us to define the normal metabolic response of both pathways to mitogens and thereby identify the precise regulatory steps that might be impaired in lymphocytes of an immunocompromised host following human immunodeficiency virus infection (1, 2, 38).

In this study, we show that de novo synthesis is essential to provide not only new RNA and DNA, but also the pyrimidine sugars necessary to support the massive expansion in membrane biosynthesis. Clear differences are also evident in the responses of pyrimidine biosynthesis to the inhibitory effects of ribavirin and azaserine in stimulated T-lymphocytes compared with those reported for malignant lymphoblasts (15, 24).

MATERIALS AND METHODS

Chemicals—Histopaque, PHA, trichloroacetic acid, and azaserine were purchased from Sigma. Lympoh-Kwik T T-cell isolation reagent was from One Lambda, Inc. (Los Angeles, CA). Ribavirin was the generous gift of ICN Pharmaceuticals (High Wycombe, Bucks, United Kingdom). RPMI 1640 medium, fetal calf serum, penicillin/streptomycin (10,000 units/ml and 10,000 μg/ml, respectively), 24-well plates, and Hank’s balanced salt solution were all obtained from Life Technologies, Inc. [14C]Hypoxanthine (0.94 mCi/ml) and [14C]glucose (0.47 mCi/ml) were purchased from Amersham plc. [3H]Uridine (0.89 mCi/ml) was from DuPont NEN.

Preparation of T-lymphocytes—Mononuclear cells were separated from heparinized blood from five healthy volunteers, diluted 1–2-fold with Hank’s balanced salt solution, and subjected to Histopaque density gradient centrifugation (2600 rpm) at room temperature. The lymphocytes were collected from the interface after centrifugation and washed once in RPMI 1640 medium. The pellet was resuspended in 1 ml of RPMI 1640 medium with 10% fetal calf serum and spun in a microcentrifuge at 1000 g for 1 min. The supernatant was removed, and T-lymphocytes were separated by incubation for 30 min at 37°C with 0.8 ml of T-cell isolation reagent (Lympoh-Kwik T). The tube was then spun at 2000 g in a microcentrifuge, the supernatant was removed, and the cell pellet was resuspended in RPMI 1640 medium and washed twice.

Stimulation of T-lymphocytes—T-cells separated as described above (58% CD4+ and CD8+ by fluorescence-activated cell sorting analysis) were counted and resuspended at 2 × 10^6 in growth medium (RPMI 1640 medium) containing physiological concentrations of phosphate (1 mm) supplemented with 10% heat-inactivated dialyzed fetal calf serum (to inactivate enzymes and to avoid the known inhibitory effects of purine and pyrimidines on de novo synthesis (7, 8)) plus antibiotics, and 0.5 ml was plated into 24-well plates.

Inhibitor Studies—Lymphocytes were preincubated with and without azaserine or ribavirin (final concentrations of 20 and 50 μM, respectively) for 30 min before adding PHA at 5 μg/ml and then incubated in...
a humidified CO₂ incubator for the periods indicated up to 72 h. Viability was measured by trypan blue exclusion.

Radioactive Tracer Studies—At 24-h intervals, cells were removed from the wells into sterile 1.5-ml Eppendorf tubes and spun at 1000 x g for 1 min. The supernatant was removed, and 100 µl of fresh medium (±inhibitor) was added plus the appropriate radiolabeled substrate (1'3H)thymidine, used to measure the flux through the purine de novo pathway, or [1'3C]xypoxanthine or [1'4]uridine, used to evaluate purine and pyrimidine salvage pathways, respectively) at a final concentration of 40 µM. The tubes were then incubated in a shaking water bath for 2 h, after which the reaction was stopped by centrifugation at 1000 x g for 1 min, and the cells were processed as described below.

Pyrimidine Ribonucleotide Pools Increase Disproportionately Relative to Purines after PHA Stimulation—The most striking observation here is that pyrimidine and purine ribonucleotide pools in healthy human T-lymphocytes expand disproportionately when stimulated by PHA. The absolute concentrations accord with the one study giving values for both in resting lymphocytes (12). The histochemical evidence, with a cutoff at 48 h (p < 0.001), and there was no increment in GTP pools (Fig. 3B; p < 0.001 at 24 h). Although there was a slight expansion in pyrimidine and pyridine nucleotide pools (Figs. 3, C–E), this was generally <30% of control, with a cutoff at 48 h (p < 0.001). Azaserine, a glutamine antagonist, is an inhibitor of several glutamine-dependent steps in nucleotide biosynthesis (15). The importance of L-glutamine in providing the amino group for the amido-transferase involved in pyrimidine and pyridine synthesis was discussed by Lyons et al. (15). However, the lack of inhibition of pyrimidine biosynthesis in their mouse leukemia cell model, but instead stimulation by azaserine, clearly differs from the up to 70% inhibition in pyrimidine pool expansion noted here.

Derangement of GTP Synthesis de Novo Is Equally Inhibitory—The effect of preincubation with ribavirin, a broad spectrum antiviral agent that inhibits de novo purine synthesis at the level of IMP dehydrogenase, the switch-point enzyme catalyzing the first step in GTP synthesis (3, 9, 16), is similar to that reported in T-lymphocytes for other IMP dehydrogenase inhibitors (3, 9). In this study, guanine nucleotide pools in T-lymphocytes preincubated with ribavirin showed no response to stimulation over 72 h (p < 0.001 at 24 h), with mean GTP concentrations being actually reduced by almost 40% compared with the resting cells (Fig. 3D). Interestingly, ATP pools at 72 h showed no change from zero time (Fig. 3, A–E, right bars; p < 0.01 compared with control), while the increment in UTP, UDP-Glc, CTP, and NAD pools at 72 h was significantly reduced compared with the control (p < 0.01, 0.05, 0.01, and 0.001, respectively). The >40% reduction in GTP pools in stimulated human T-lymphocytes is similar to that reported for ribavirin in mouse lymphoma cells (18). However, the inhibitory effect of ribavirin evident here on pyrimidine pool expansion (~60% of control at 72 h) is in complete contrast to the substantial increase in CTP and UTP pools (150 and 170% of control, respectively) accompanying the 60% decrease in GTP pools reported in mouse L5178Y cells incubated in the presence of

In the HPLC method used, dCTP, dATP, and dGTP have retention times between 0.7 and 1 min later than the corresponding ribonucleoside triphosphate (limit of detection, 1 pmol/10⁶ cells) (Fig. 2B), although dTTP elutes on the front of GTP. The chromatogram showing the different nucleotides present in healthy lymphocytes at 72 h (Fig. 2A) was automatically autoscaled to the highest peak (ATP) to show the individual UV spectra by in-line photodiode array. However, peaks were quantified at 0.02 absorbance units full scale. Thus, any significant increments in dNTPs in cellular lymphocyte extracts following PHA stimulation, as reported by others using enzyme assays (19–21, 25), should have been detectable. The advantages and disadvantages of HPLC compared with the enzyme assay have been reviewed (20). The absence of detectable increments in dNTP pools here, coupled with the fact that DNA synthesis increases markedly after stimulation, suggests that these pools must be highly localized in order to be at sufficiently high concentrations to drive DNA synthesis. The findings support studies postulating the existence of multienzyme complexes that both synthesize dNTPs and channel them to sites of DNA replication (26).

Purine and Pyrimidine Synthesis de Novo Is Essential for T-lymphocytes to Complete the Cell Cycle—The importance of de novo synthetic pathways to enable the ribonucleotide pools in resting T-lymphocytes from immunocompetent donors to undergo such dramatic increments in response to PHA is clearly demonstrated by preincubation with azaserine (Fig. 3, A–E, center bars). In these T-lymphocytes from healthy humans, the inhibitory effect of azaserine was evident from 24 h onward, being maximal by 48 h. ATP pools were actually reduced to 50% of control over 72 h (Fig. 3A; p < 0.001), and there was no increment in GTP pools (Fig. 3B; p < 0.001 at 24 h). Although there was a slight expansion in pyrimidine and pyridine nucleotide pools (Figs. 3, C–E), this was generally <30% of control, with a cutoff at 48 h (p < 0.001). Azaserine, a glutamine antagonist, is an inhibitor of several glutamine-dependent steps in nucleotide biosynthesis (15). The importance of L-glutamine in providing the amino group for the amidotransferases involved in purine and pyrimidine synthesis was discussed by Lyons et al. (15). However, the lack of inhibition of pyrimidine biosynthesis in their mouse leukemia cell model, instead stimulation by azaserine, clearly differs from the up to 70% inhibition in pyrimidine pool expansion noted here.

RESULTS

Pyrimidine Ribonucleotide Pools Increase Disproportionately Relative to Purines after PHA Stimulation—The most striking observation here is that pyrimidine and purine ribonucleotide pools in healthy human T-lymphocytes expand disproportionately when stimulated by PHA. The absolute concentrations accord with the one study giving values for both in resting T-lymphocytes and at 40 h after stimulation (12). The histograms (Fig. 3) demonstrate that mean control purine ribonucleotide concentrations (adenine nucleotides (ATP, ADP, and AMP; Fig. 3A) and guanine nucleotides (GTP and GDP; Fig. 3B)) increased up to 2-fold and up to 7-fold for the pyridine nucleotide NAD and ADP-ribose (Fig. 3E). However, the greatest increment is in pyrimidine nucleotides UTP, UDP, and UDP-Glc (up to 8-fold; Fig. 3C) and CTP (Fig. 3D) 72 h after PHA stimulation. The triphosphates (hatched or dotted sections) were always the predominant nucleotide present and exceeded the diphosphates (black sections) manifold, indicating the excellent condition and high energy charge of the cells throughout the 72-h period. This was supported by trypan blue exclusion (viability > 90%). CDP concentrations (not shown) were always very low relative to CTP (~1.50), and no deoxyribonucleotides (dNTPs) were detected either in extracts of resting T-lymphocytes or at any time over the 72 h following PHA stimulation.
of ribavirin (24).

Distribution of Radiolabeled Precursors Confirms Importance of de Novo Synthesis—Studies in control cells (Fig. 4, A–D, left bars) pulse-labeled with $[14^C]$glycine for 2 h show that the de novo purine synthetic activity is undetectable in resting T-cells, but clear stimulation is evident from 24 h, with the incorporation into ATP greatly exceeding that into GTP, as reported by others (11, 12). The importance of intact de novo pathways for purine ribonucleotide synthesis is illustrated by the studies in cultures exposed to azaserine. Glycine incorporation into both ATP and GTP was completely blocked (Fig. 4, A and B, center bars). Radiolabel was found exclusively in the de novo intermediates, formylglycinamide ribotide and its di- and triphosphates (Fig. 4C), demonstrating inhibition at the fourth step of the de novo route (Fig. 1) catalyzed by formylglycine-amidinase synthetase, as reported in azaserine-inhibited leukemia cells (15). Ribavirin (Fig. 4, A–D, right bars) selectively inhibited glycine incorporation into GTP, but not into ATP (Fig. 4, A and B), with some accumulation of IMP (Fig. 4C), confirming inhibition of GTP synthesis at the level of IMP dehydrogenase.

The linear increment in the incorporation of radiolabel into the cell precipitate (Fig. 4D) confirms accelerated synthesis of RNA, DNA, and protein over 72 h in control cells (left bars), which is arrested at 24 h by azaserine (center bars) and at 48 h by ribavirin (right bars). The evident reduction in radiolabel incorporation into the cell precipitate after 24 h in cells incubated with azaserine and after 48 h with ribavirin compared
with the control is consistent with cell cycle arrest. This interpretation is in accord with reports showing inhibition of DNA synthesis in the presence of these antagonists at the G1 and G1/early S transition phases, respectively (3, 15, 18). This assumption is supported by the finding that although both drugs inhibited cell growth in our experiments, cell viability (trypan blue exclusion) was unaffected.

**Purine and Pyrimidine Salvage Is Also Stimulated by PHA**—In contrast to the above [14C]glycine studies, which confirm little de novo purine activity in resting lymphocytes, significant salvage activity was evident for purines ([14C]hypoxanthine) (Fig. 5, A and B), but salvage was much less for pyrimidines ([14C]uridine) (Fig. 5, C and D). The exponential increase in the incorporation of hypoxanthine into ATP and especially GTP or of uridine into UTP, UDP-Glc, and, to a lesser extent, CTP indicates that salvage pathways are also stimulated by PHA. Studies (not shown) evaluated the incorporation of radiolabeled aspartic acid and orotic acid (intermediates in the de novo pyrimidine pathway). While both were taken up, there was no significant incorporation into soluble nucleotides, which is similar to previously reported results (9). Other precursors are being investigated.

**DISCUSSION**

In this study, we demonstrate that initiation of blast transformation in T-lymphocytes from healthy subjects requires extensive activation of housekeeping genes coding for key purine and pyrimidine enzymes involving both salvage and particularly the de novo synthetic routes of ribonucleotide formation (Fig. 1). There are several noteworthy observations.
First, the anticipated expansion in both pyrimidine and purine ribonucleotide pools is evident at 72 h following PHA stimulation, but the relative increment in pyrimidine pools is much greater than for purines. The disproportionate increment in pyrimidine ribonucleotide concentrations in stimulated T-lymphocytes in this study occurred in the absence of exogenous pyrimidine ribonucleosides and included the UDP-sugars. This finding supports an additional role for de novo pyrimidine synthesis in T-lymphocyte proliferation, namely, to provide the extra pyrimidine ribonucleotides necessary for the massive expansion in membrane biosynthesis. The mechanism underlying this stimulation could be enhanced expression of the multifunctional protein CAD (which contains the activities of carbamoyl-phosphate synthetase II, aspartate carbamoyltransferase, and dihydroorotase), as reported for stimulated murine B-cells (27). In that study (27), modest increases over 12 h preceded a profound increase from 24 to 60 h, which correlated with movement from the G1 to the S phase of the cycle. Metabolic experiments by others using labeled mannose and choline in activated murine B-lymphocytes have shown a dramatic phase-specific induction of dolichol-linked lipid intermediate synthesis, protein N-glycosylation activity, and phospholipid synthesis (28, 29). Interestingly, the incorporation of radiolabeled choline was reduced drastically by an inhibitor of CDP-choline synthetase (30). Obviously, any clinical situation reducing the supply of CTP (the essential substrate for this enzyme), as in the inhibitor studies discussed below or in the human immunodeficiency virus type-1 T-lymphocyte experiments in the accompanying paper (38), would have a similar effect.

Second, the radiotracer studies comparing the routes of ribonucleotide formation confirm that resting lymphocytes meet their metabolic requirements by salvage, with the de novo purine pathway being virtually inactive (11, 12). Uridine salvage, although relatively inactive in resting lymphocytes, is also stimulated, particularly at 48 h, confirming uridine kinase as another enzyme induced during the S phase (31). The limited conversion of uridine into CTP accords with the recent report that uridine salvage is not a significant source of CTP in stimulated lymphocytes, although it is the preferred route in malignant human T-lymphocytic cells (32). Other studies demonstrating active uptake of exogenous dCyd and CdC into phosphatidylcholine, via dCDP-choline and CDP-choline and the corresponding diacylglycerol intermediates, imply a special role for pyrimidine salvage in phosphatidylinositol synthesis (33). These observations are in agreement with earlier reports proposing the existence of independent pyrimidine pools (5, 26).

Third, the considerable expansion in the NAD pool evident here in stimulated T-lymphocytes reflects the requirement of dividing lymphocytes for an additional supply of NAD for a number of NAD-dependent reactions, such as the repair of spontaneous DNA strand breaks (34, 35). Poly(ADP-ribose) polymerase works only in the presence of NAD (16). The inhibitory effect of azaserine on NAD synthesis supports inhibition of the glutamine-dependent NAD synthetase. The importance of NAD to proliferating lymphocytes is underlined by the fact that NAD depletion, activated by DNA strand breaks, has been implicated in the lymphotoxicity in adenosine deaminase deficiency (6, 8).
Fourth, the finding that azaserine clearly inhibits expansion of both purine and pyrimidine nucleotide pools provides the first direct evidence of the importance of intact de novo synthetic pathways to blasting lymphocytes. However, the inhibitory effect on both pathways is contrary to the belief that potent inhibition of either de novo purine or pyrimidine biosynthesis is accompanied by a complementary stimulation of the flux through the other pathway (15). Inhibition of purine synthesis de novo at the level of formylglycinamide ribotide, together with its di- and triphosphates, as also noted in mouse leukemia cells (15). The reduction in uridine and cytidine ribonucleotide pools is consistent with a block in pyrimidine de novo synthesis at the level of carbamoyl-phosphate synthetase II and CTP synthetase (Fig. 1), as reported for the anti-glutamine agent acivicin in malignant cells in vitro (15). The inhibition of pyrimidine de novo synthesis by azaserine is evident from the fluorescence-activated cell sorting analysis of T-cell subsets. The vital role of IMP dehydrogenase in GTP synthesis for blasting T-lymphocytes, evident from the depletion induced by ribavirin, is well documented for other IMP dehydrogenase inhibitors (3, 9, 16, 18). GTP depletion could also impair functions other than DNA synthesis in proliferating T-lymphocytes, e.g., the activity of G-proteins involved in signal transduction or formation of the GDP-mannose and GDP-fucose intermediates essential for the glycosylation of adhesion molecules. Such a dual effect may explain the enhanced efficacy of IMP dehydrogenase inhibitors, currently in clinical trial for organ transplantation and cancer chemotherapy (3, 16).

The results presented here question the concept of coordinate regulation of purine and pyrimidine biosynthesis, which putatively equalizes the rates of formation of purine and pyrimidine nucleotides for nucleic acid synthesis. The findings support the suggested metabolic channeling of dNTPs (26) and highlight the particular importance of pyrimidine ribonucleotide availability to mitogen-stimulated T-lymphocytes. The significance of this in vivo for human immunodeficiency virus type-1-infected lymphocytes is discussed in the accompanying paper (38). The marked differences evident here in the regulation of nucleotide biosynthesis between dividing human T-lymphocytes and malignant lymphoid cells (15, 24) suggest new avenues that could be exploited therapeutically.

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