The Influence of Ribose 5-Phosphate Availability on Purine Synthesis of Cultured Human Lymphoblasts and Mitogen-stimulated Lymphocytes*

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The intracellular ribose 5-phosphate concentration was found to be an important determinant of rates of de novo purine synthesis. When ribose 5-phosphate production was reduced in cultured human lymphoblasts by glucose starvation, the intracellular phosphoribosylpyrophosphate concentration and rates of de novo purine synthesis decreased. Inosinate-guanulate:pyrophosphate phosphoribosyltransferase (HPR transferase)-deficient cells were relatively more resistant to glucose starvation. To minimize the effect of purine nucleotide feedback inhibition on the de novo pathway, cells were treated with inhibitors of IMP dehydrogenase and adenylosuccinate synthetase. In normal lymphoblasts, purine synthesis was stimulated only at glucose concentrations greater than 100 μM while in HPR transferase-deficient lymphoblasts, stimulation occurred even at low glucose concentrations. The differences between the normal and HPR transferase-deficient cells were lost when ribose reutilization from endogenous nucleotide breakdown was impaired in the HPR transferase-deficient cells by incubation with 2′-deoxyinosine. Endogenous ribose reutilization for purine synthesis is, therefore, important when either glucose availability is limited or synthesis is stimulated. In the absence of glucose, exogenous purine nucleotides restored the intracellular concentrations of ribose 5-phosphate, phosphoribosylpyrophosphate, and purine nucleotides to almost 100% and rates of purine synthesis to 50–75% of those at 10 mM glucose. When ribose 5-phosphate production was increased by phytohemagglutinin activation, the intracellular phosphoribosylpyrophosphate concentration and rates of de novo purine synthesis increased.

PP-Rib-P1 plays a critical role in the regulation of de novo purine synthesis (1–4). It serves as both substrate and activator of amidophosphoribosyltransferase (glutamine phosphoribosylpyrophosphate amidotransferase, EC 2.4.2.14), the first and presumed rate-determining enzyme of de novo purine synthesis (5) (Fig. 1). In intact cells increased availability of PP-Rib-P correlates with enhanced de novo purine synthesis (6–8) and decreased availability of PP-Rib-P with reduced de novo purine synthesis (9–11). However, some workers have been unable to demonstrate such correlations (12).

Rib-5-P, the precursor of PP-Rib-P, has also been proposed to be an important factor controlling the rate of de novo purine synthesis, but this has never been established (1–3, 13). That increased Rib-5-P availability leads to purine overproduction is suggested by three genetic disorders which cause hyperuricemia in man. The first is a mutant PP-Rib-P synthetase (ATP:a-D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) with increased affinity for Rib-5-P; this effectively increases the kinetic availability of Rib-5-P (14). The other two are glucose 6-phosphatase deficiency (15) and increased activity of glutathione reductase (16); both mutations are thought to increase Rib-5-P generation by increasing the flux through the pentose phosphate pathway (3) (Fig. 1). The experimental decrease of the NADPH/NADP ratio induced by methylene blue or other electron acceptors significantly increases the intracellular concentrations of Rib-5-P and PP-Rib-P and stimulates de novo purine synthesis (6–8).

We chose the following experimental approaches to explore the influence of Rib-5-P availability on the rate of de novo purine synthesis in cultured human lymphoblasts: 1) starvation for glucose to limit the Rib-5-P generation through the pentose phosphate pathway; 2) incubation with purine nucleosides to serve as alternative Rib-5-P sources; and 3) inhibition of purine nucleoside phosphorylase (purine nucleoside:phosphoribosyl transferase, EC 2.4.2.1) to prevent endogenous ribose reutilization from intracellular nucleotide breakdown (Fig. 1). The above studies were performed with de novo purine synthesis under normal feedback control by purine nucleotides and with feedback inhibition released by a combination of drugs that decrease the formation of adenylates and guanylates from IMP. The latter condition allowed us to investigate the Rib-5-P requirement for maximally stimulated de novo purine synthesis. We also used mitogen-activated peripheral blood lymphocytes as a model for stimulated purine synthesis and measured the specific activities of PP-Rib-P synthetase and amidophosphoribosyltransferase, and the intracellular concentrations of Rib-5-P, PP-Rib-P, purine nucleotides, and inorganic phosphate. We found that the intracellular Rib-5-P concentration can be rate-determining for de novo purine synthesis.
EXPERIMENTAL PROCEDURES

RESULTS

Effect of Glucose Starvation on de Novo Purine Synthesis in Cultured Lymphoblasts—When normal or HPR transferase-deficient lymphoblasts were transferred to glucose-deficient media, rates of de novo purine synthesis stabilized in the normal and HPR transferase-deficient lymphoblasts at 7 ± 2 and 14 ± 3%, respectively, of the control values measured in the presence of 10 mM glucose. Addition of 10 mM glucose to either of the cell types at any time during a 6-h glucose starvation resulted in a rapid (within 15 min) and a complete restoration of de novo purine synthesis. Maximal rates of de novo purine synthesis occurred at 10 mM glucose in both cell types, but half-maximal rates of de novo purine synthesis occurred at greater than 500 μM glucose in the normal cells and 100 μM glucose in the HPR transferase-deficient cells (Figs. 2 and 3, respectively, open circles).

Effect of Mycophenolic Acid, Alanosine, and Hadacidin on de Novo Purine Synthesis during Glucose Starvation in Cultured Lymphoblasts—To eliminate the control of de novo purine synthesis by purine nucleotide feedback inhibition (3-5), we incubated lymphoblasts with the combination of mycophenolic acid, alanosine, and hadacidin. We previously demonstrated (27) and further show below that these drugs decrease the intracellular concentration of purine nucleotides and cause a stimulation of de novo purine synthesis. In addition to release of feedback inhibition, the drugs also increase the reutilization of the ribose moiety of purine nucleotides (see below). In the normal lymphoblasts, de novo purine synthesis was stimulated by the drugs only at glucose concentrations greater than 100 μM (Fig. 2, closed circles).

In the HPR transferase-deficient lymphoblasts, on the other hand, de novo purine synthesis was stimulated significantly (p < 0.05, Student’s t test) by the drugs at all concentrations of glucose and even in its absence (Fig. 3, closed circles). In both cell types, the degree of stimulation increased as the glucose concentration increased; stimulation was maximal at 10 mM glucose in the normal lymphoblasts and at 1 mM glucose in the HPR transferase-deficient lymphoblasts.

Effect of Alternative Ribose Sources on de Novo Purine Synthesis in Cultured Lymphoblasts—Since lymphoblasts lack ribokinase and are not readily permeable to ribose phosphates, we used purine nucleosides to function as alternative ribose phosphate sources. Inosine, guanosine, and their analogs are readily transported inside the cell and are then phosphorylated by purine nucleoside phosphorylase. This phosphorylation yields the purine base and Rib-1-P which is converted to Rib-5-P by phosphoribomutase (Fig. 1).

We used 8-aminoguanosine as the alternative ribose source for normal lymphoblasts. A disadvantage of 8-aminoguanosine is that this nucleoside is a relatively poor substrate of purine nucleoside phosphorylase (33). However, the advantage of this compound is that the product, 8-aminoguanine, is not a substrate for HPR transferase and, therefore, cannot lead to the production of a nucleotide inhibitor of PP-Rib-P synthetase or amidophosphoribosyltransferase (34). In the absence of glucose, 50 μM 8-aminoguanosine increased de novo purine synthesis of normal lymphoblasts almost 8-fold (compare bars a and b in the absence of glucose, inset, Fig. 2) and provided sufficient ribose to the lymphoblasts so that they further increased their rates of de novo purine synthesis in response to mycophenolic acid, alanosine, and hadacidin (compare bars c and d, inset, Fig. 2). In the presence of 100 μM glucose, 8-aminoguanosine increased de novo purine synthesis significantly only in the presence of mycophenolic acid, alanosine, and hadacidin (bar d at 100 μM glucose, inset, Fig. 2). At 10 mM glucose, the addition of 8-aminoguanosine had no significant effect.

HPR transferase-deficient lymphoblasts are unable to salvage hypoxanthine and, therefore, afford the opportunity to provide Rib-5-P to the cell from the preferred purine nucleoside phosphorylase substrate, inosine, without producing purine nucleotides (Fig. 4). At concentrations below 30 μM, inosine increased de novo purine synthesis more than did glucose in the absence or presence of mycophenolic acid,
alanosine, and hadacidin. The maximal rate of de novo purine synthesis was achieved with 30 μM to 1 mM inosine in the absence of the drugs and was 55% of the rate achieved with 1–10 mM glucose. However, in the presence of the drugs, the maximal rate of de novo purine synthesis was achieved with 1–3 mM inosine and was about 70% of the rate achieved with 1–10 mM glucose. These differences between glucose and inosine were still observed when the experimental protocol was modified and glucose and inosine were simultaneously added to parallel cultures.

Effect of Purine Nucleoside Phosphorylase Inhibition on de Novo Purine Synthesis in Cultured Lymphoblasts—Inhibition of purine nucleoside phosphorylase activity should allow one to determine the contribution of endogenous ribose reutilization to the Rib-5-P and PP-Rib-P available for de novo purine synthesis (Fig. 1). We used 2′-deoxyinosine to inhibit the phosphohydrolysis of inosine by purine nucleoside phosphorylase in HPR transferase-deficient lymphoblasts. While more potent purine nucleoside phosphorylase inhibitors are available, they all can either serve to some degree as a ribose source or function as inhibitors, of de novo purine synthesis (35). 2′-Deoxyinosine is hydrolyzed by purine nucleoside phosphorylase, but the resulting 2′-deoxy-Rib-1-P cannot serve as a precursor of PP-Rib-P and as before, the HPR transferase-deficient cells cannot salvage the hypoxanthine.

In either the absence of glucose or in the presence of 100 μM glucose, [14C]formate incorporation was significantly reduced by 100 μM 2′-deoxyinosine (compare bars a and b in the absence of glucose and at 100 μM glucose, inset, Fig. 3). This suppression was due neither to 2′-deoxy-Rib-1-P toxicity nor to nucleotide inhibition via residual HPR transferase activity, since it could be completely reversed by the addition of 100 μM inosine. In the presence of 10 mM glucose, 2′-deoxyinosine had no effect. When de novo purine synthesis of HPR transferase-deficient lymphoblasts was stimulated by mycophenolic acid, alanosine, and hadacidin, the addition of 2′-deoxyinosine significantly decreased the rate of [14C]formate incorporation irrespective of the glucose concentration (compare bars c and d at all three glucose concentrations, inset, Fig. 3). The most pronounced effect of 2′-deoxyinosine was seen in the absence of glucose where it prevented any stimulation of de novo purine synthesis by the drugs. These results indicate that the contribution of endogenous ribose reutilization to the Rib-5-P available for de novo purine synthesis is significant when 1) the supply of Rib-5-P from the pentose phosphate pathway is reduced because of glucose starvation, or 2) de novo purine synthesis is stimulated by mycophenolic acid, alanosine, and hadacidin.

Effect of Glucose Starvation on the Concentration of Purine Nucleotides in Cultured Lymphoblasts—When normal lymphoblasts were incubated in the glucose-deficient media for 24 h, the intracellular GTP, ATP, and ADP concentrations decreased by 43, 40, and 30%, respectively, as compared to control values measured in the presence of 10 mM glucose; the GDP concentration remained unchanged (Table 1). We
were unable to detect major changes, greater than 2-fold, in the intracellular concentrations of AMP, GMP, and IMP during glucose starvation (data not shown). Results obtained with HPR transferase-deficient lymphoblasts were virtually identical. When inosine was added to glucose-starved HPR transferase-deficient cells the intracellular concentrations of purine nucleotides were similar to those at 10 mM glucose (data not shown).

Effect of Mycophenolic Acid, Alanosine, and Hadacidin on the Concentration of Purine Nucleotides in Cultured Lymphoblasts—When normal lymphoblasts were incubated for 2% h in the presence of 10 mM glucose with mycophenolic acid, alanosine, and hadacidin, the intracellular concentration of purine nucleotides decreased (Table I). Compared to control cultures without the drugs, the intracellular ATP and ADP concentrations decreased by about 21 and 38%, respectively, while the intracellular GTP and GDP concentrations decreased by about 74 and 44%, respectively. When the drugs were added to glucose-starved lymphoblasts, they did not decrease the concentration of adenylate any further, while GTP and GDP decreased by 28 and 17%, as compared to glucose-starved cultures without the drugs. When the drugs were added to cells in 0.01, 0.1, and 1 mM glucose, intermediate changes between those in the absence of glucose were virtually identical. When inosine was added to glucose-starved HPR transferase-deficient lymphoblasts were incubated at 10 mM glucose, the drugs caused a significant increase in the Rib-5-P and PP-Rib-P concentrations and [14C]adenine incorporation (Table II). These results correlated with the lack of stimulation of de novo purine synthesis by the drugs under these conditions. When HPR transferase-deficient lymphoblasts were incubated under the same conditions, the drugs caused a significant increase in the Rib-5-P and PP-Rib-P concentrations and [14C]adenine incorporation; 2'-deoxyinosine prevented this increase (Table III). Again, these results correlated with the rates of de novo purine synthesis seen in glucose-starved HPR transferase-deficient lymphoblasts: mycophenolic acid, alanosine, and hadacidin caused a 2.4-fold stimulation which was prevented by 2'-deoxyinosine.

When normal and HPR transferase-deficient lymphoblasts were then incubated at glucose concentrations that allowed half-maximal rates of de novo purine synthesis, mycophenolic acid, alanosine, and hadacidin increased the Rib-5-P and PP-Rib-P concentrations and [14C]adenine incorporation to values that were markedly above the values measured with 10 mM glucose in the absence of the drugs. Finally, when lymphoblasts were incubated at 10 mM glucose, the drugs caused a more than 4-fold increase in the Rib-5-P and PP-Rib-P concentrations and [14C]adenine incorporation in both cell types. However, the stimulation of de novo purine synthesis under these conditions was only 2-2.5-fold.

DISCUSSION

In this study, we demonstrated that the intracellular concentration of Rib-5-P, the precursor of PP-Rib-P, can be limiting for the rate of de novo purine synthesis. When the intracellular Rib-5-P concentration was decreased in cultured lymphoblasts by glucose starvation, the intracellular PP-Rib-P concentration and rates of de novo purine synthesis decreased. Moreover, when the intracellular Rib-5-P concentration was increased in peripheral blood lymphocytes by mitogen activation, the intracellular PP-Rib-P concentration and rates of de novo purine synthesis increased. This latter increase of purine synthesis could not be attributed to an increase in the specific activities of either PP-Rib-P synthetase or amidophosphoribosyltransferase or to changes in the intracellular concentration of phosphate or purine nucleotides.

When HPR transferase-deficient lymphoblasts were compared to normal lymphoblasts, the deficient cells showed increased resistance to glucose starvation: they maintained higher rates of de novo synthesis in the absence of glucose and required less glucose for half-maximal rates. The difference between the two cell types may be explained by increased PP-Rib-P availability in the HPR transferase-deficient cells which is documented by an increased intracellular PP-Rib-P concentration and increased [14C]adenine incorporation. This increased PP-Rib-P availability in the HPR transferase-deficient cells is because of ribose salvage from endogenous nucleotide breakdown in the absence of hypoxanthine salvage, i.e. unilateral ribose reutilization (42, 45). The contribution of this ribose reutilization to Rib-5-P availability for de novo purine synthesis was studied in the HPR transferase-deficient
cells by treating them with 2′-deoxyinosine, an alternative substrate for purine nucleoside phosphorylase that does not yield Rib-5-P (39). We found that the reutilization of ribose from endogenous nucleotide breakdown becomes a significant factor for the maintenance of de novo purine synthesis at low glucose concentrations (<100 μM), whereas in the presence of higher glucose concentrations, ribose reutilization does not seem to be necessary for normal rates of de novo purine synthesis.

We determined the degree to which purine nucleosides can replace glucose as a ribose source for de novo purine synthesis. On a molar basis, low concentrations of 8-aminoguanosine in normal lymphoblasts and inosine in HPR transferase-deficient lymphoblasts were more efficient than glucose in increasing Rib-5-P and PP-Rib-P concentrations, as well as rates of de novo purine synthesis. However, higher concentrations of nucleosides were less effective and neither nucleoside could fully replace glucose; maximal rates of de novo purine synthesis achieved in the presence of 8-aminoguanosine or inosine were about 55% of those observed in the presence of 10 mM glucose. Comparably high intracellular Rib-5-P and PP-Rib-P concentrations were established in the presence of inosine and glucose. This eliminates the possibility that the rate of nucleoside transport or the conversion by purine nucleoside phosphorylase and phosphoribomutase might have been rate-limiting under these conditions. Inhibition of de novo purine synthesis by residual HPR transferase activity and consequent nucleotide formation is also excluded since the concentration of purine nucleotides in the presence of 1 mM inosine were the same as in the presence of 10 mM glucose and maximal, feedback-released de novo purine synthesis in the presence of mycophenolic acid, alanosine, and hadacidin with 1 mM inosine was still only 70% of that observed with 10 mM glucose in the presence of the drugs. We cannot exclude the possibility of some other unidentified side effect of high nucleoside concentrations on the de novo pathway. A more plausible explanation is that glucose metabolism provides a factor in addition to Rib-5-P that is necessary for maximal rates of de novo purine synthesis.

The control of de novo purine synthesis is complex and feedback inhibition by purine nucleotides is a major determinant (3–5, 43). The intracellular concentrations of GTP, ATP, and ADP decreased during glucose starvation without major changes in the purine nucleoside monophosphate pools. A partial release of feedback inhibition on PP-Rib-P synthetase and amidoribosyltransferase in glucose-starved cells is, therefore, possible (6, 43). We felt it necessary to eliminate the control of de novo purine synthesis by feedback inhibition in order to fully evaluate the influence of Rib-5-P and PP-Rib-P availability on the rate of de novo purine synthesis. The combination of mycophenolic acid, alanosine, and hadacidin prevents the synthesis of guanylates and adenylates from IMP, thereby depleting the intracellular nucleotide pools and releasing feedback inhibition (22, 24, 27). We found that in normal lymphoblasts de novo purine synthesis could be stimulated by the drugs only at glucose concentrations greater than 100 μM, whereas in HPR transferase-deficient lymphoblasts, stimulation was observed even in the absence of glucose. The changes in the concentration of purine nucleotides during glucose starvation and incubation with the drugs were virtually identical in both cell types. Again, the difference between the two cell types can be explained by the increased PP-Rib-P availability in the HPR transferase-deficient cells because of unilateral ribose reutilization (42, 45). In addition to release of feedback inhibition, mycophenolic acid, alanosine, and hadacidin increase ribose reutilization. Under normal conditions, cytosolic 5′-nucleotidase competes with IMP-dehydrogenase and adenylosuccinate synthetase for the substrate IMP (Fig. 1). Previous studies indicate that as much as 30–50% of newly synthesized IMP is subject to degradation and participates in the “inosinate cycle” (44, 45). This cycle is composed of cytosolic 5′-nucleotidase, purine nucleoside phosphorylase, phosphoribomutase, PP-Rib-P synthetase, and HPR transferase (Fig. 1). In the presence of inhibitors of IMP-dehydrogenase and adenylosuccinate synthetase, the only possible fate of the accumulating IMP is degradation. Thus, the flux through the inosinate cycle is greatly enhanced. Hypoxanthine that is generated can be excreted into the media, whereas the ribose moiety is salvaged as ribose phosphate, retained within the cell, and reutilized. Indeed, in the presence of mycophenolic acid, alanosine, and hadacidin, greater than 90% of the total de novo synthesized purines were found in the culture media and the intracellular Rib-5-P and PP-Rib-P concentrations were markedly increased.

Because mycophenolic acid, alanosine, and hadacidin increase the flux through the inosinate cycle and increase Rib-5-P reutilization, de novo purine synthesis in the presence of the drugs may require less ribose from the pentose phosphate pathway than without the drugs. We should note that we observed ribose reutilization for purine synthesis, but we do not know how much ribose may also be used for synthesis of other compounds, for example, lactic acid. The importance of ribose reutilization for the stimulation of de novo purine synthesis by mycophenolic acid, alanosine, and hadacidin was shown in HPR transferase-deficient cells, since 2′-deoxyinosine prevented the stimulation at glucose concentrations of 100 μM or less. Again, 100 μM glucose seems to be a critical concentration for de novo purine synthesis. Below this concentration, de novo purine synthesis cannot be increased in response to released feedback inhibition unless additional Rib-5-P and PP-Rib-P are provided by endogenous ribose reutilization in the absence of hypoxanthine salvage (HPR transferase deficiency) or by an alternative exogenous ribose source. Even at 10 mM glucose, stimulation of de novo purine synthesis by the drugs was reduced in the presence of 2′-deoxyinosine. The importance of ribose reutilization for the stimulation of de novo purine synthesis by mycophenolic acid, alanosine, and hadacidin also has been shown in studies with purine nucleoside phosphorylase-deficient lymphoblasts (46).

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Ribose 5-Phosphate and Purine Synthesis

SUPPORT MATERIAL TO:
THE INFLUENCE OF RIBOSE-5-PHOSPHATE AVAILABILITY ON PURINE SYNTHESIS OF CULTURED HUMAN LYMPHOCYTES AND MITOGEN-STIMULATED LYMPHOCYTES

by
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Fig. 1. In vivo Purine Nucleoside Synthesis and Metabolism Pathway. The reactions shown are: (1) ribose-5-phosphate pentose pathway; (2) phosphoribosylpyrophosphate synthetase, (3) phosphoribosylimidazolecarboxylase; (4) phosphoribosyltransferase; (5, 6) IMP synthetase and deaminase; (7) ATP synthetase and deaminase; (8) PRPP synthetase. The enzymes involved are: (1) ribokinase, (2, 3) glutamine phosphoribosyltransferase, (4, 5, 8) aspartate transcarbamylase, (6) adenine phosphoribosyltransferase, (7) IMP synthetase and deaminase. The site of action of phosphoribosylpyrophosphate is denoted by PRPP, the site of action of adenosine is denoted by AMP, and of inosine is denoted by IMP.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Lymphocytes

The experimental culture medium for the lymphoblasts was prepared from Dulbecco's modified Eagle's medium deficient in glucose, pyruvate, glutamine, and bicarbonate buffer (Dulbecco Scientific, Irvine, CA) that was supplemented with 25 mM HEPES, pH 7.3, and 20 mM NaHCO3. In addition, the following supplements were added to 1% of the medium: 25 µg/mL insulin, 25 µg/mL transferrin, 25 µg/mL hydrocortisone, 25 µg/mL selenium, 10 µg/mL insulin-like growth factor (IGF-I, Sigma), 2 ng/mL phytohemagglutinin (PHA, Sigma), 1% of fetal bovine serum (FBS, Sigma), 1% nonessential amino acids (Sigma), 1% penicillin-streptomycin, and 220 µM F-68 (polyvinyl alcohol, Sigma). The final cell concentration was 1 x 10^6 cells/mL and the medium was exchanged daily.

Preparation of Glucose-Deficient Media

For the preparation of cultures, lymphocytes were suspended in the glucose-free medium at 1 x 10^6 to 1.2 x 10^6 cells/mL. They were then transferred to either 15 or 35 mm tissue culture dishes for incubation for 24 or 48 h, respectively.

Preparation of Mitomycin C

Mitomycin C was prepared as a 50 mg/mL solution in saline and diluted with medium to the desired concentration.

Measurement of RIB5P and PPRibP

Electronic Cell Volumetry

The measurement of the lymphocytes was determined by electronic analysis using a Coulter Counter (Model, CO). After thorough resuscitation of the lymphocytes, 0.5 mL of cell solution was diluted with 4.5 mL of medium containing the following settings: aperture 0.2 µm, aperture current 1.0, aperture 0.2 nm, lower threshold 10 µm, upper threshold 100 µm. The electronic cell counter was calibrated with 100,000 cells/mL and a standard deviation of 0.1% or less. The carrier DMSO was used to accurately determine the number of cells in the sample. The lower threshold [7] was used for calculation of the cell volume as described in the Coulter Counter Manual.

Measurement of Incorporation into the Lymphocyte Culture Media

All of the lymphocyte culture media were extracted in 0.4 M perchloric acid and neutralized with barium hydroxide as above. The isotope was quantified by high performance liquid chromatography on a 25 cm Beckman reverse phase column (5 µm, using 3% acetonitrile).

Measurement of Lymphocytes

Ribose 5-phosphate synthesis was determined by the method of Roux, et al [30]. First, the lymphocytes were cultured in the presence of [1-14C]ribose 5-phosphate (SPR, 50 Ci/mmol) and, after 24 h, cells were collected and washed twice with 5 mL of cold phosphate buffer. The cells were then digested with 0.3 mL of 70% H2SO4, 20 min at 100°C. After cooling and dilution to 10 mL with water, 0.2 mL of each sample was spotted on anion exchange paper (Whatman 3MM). The paper was developed in 1 M sodium acetate buffer and stained with 0.2% Alizarin S. After development, the areas corresponding to 14C radioactivity were excised and counted in a toluene-based scintillation cocktail.

Finally, de novo purine synthesis was calculated from the sum of radiotracer pulses in the cells and in the medium. Since ferric serves as a tracer and it is not known whether 1, 2, or 3 of the purine nucleosides exported to the extracellular medium, the following assumptions were made: (1) that all 3 purine nucleosides are exported, (2) that only de novo purine synthesis is exporting to the extracellular medium, and (3) that only 1 purine nucleoside is exported.

The assay was performed in duplicate with individual values differing by less than 10% and was measured from 0.1 to 2.0 x 10^6 cells for up to 24 h.

Measurement of Purine Nucleotides

After a 24 h incubation period, 20-30 x 10^6 cells were cooled on ice and washed in 3 x 106 of a buffer that contained 150 mL NaCl, 20 mL HEPES, pH 7.4 (Calbiochem, La Jolla, CA), 0.1 mL CaCl2, and 0.1 mL MgCl2. An 18 s protein concentration of 5 mg/mL was used at a density of 1 x 10^6 in 0.4 M perchloric acid and the supernatant recovered to two times the original volume of phosphate buffer. The supernatant was then recovered by heating at 95°C for 30 s and heating at 20°C for 15 s. After a 30 min wash with 100 mL of 0.5 M KCl (buffer B), after 5 h of a buffer B, R buffer is linearly increased to 1 M KCl over the next 1.5 h, and it is held at 1 M KCl for 15 min. Individual nucleotide assays were performed by comparison to an appropriate standard. The concentration of PRPP was determined by HPLC.
### Table I

**Intracellular Concentration of Purine Nucleotides in Normal Lymphoblasts**

Normal lymphoblasts were transferred to glucose-deficient media. Variable amounts of glucose and/or 2- deoxyinosinic acid, 2'-deoxyadenosine, and/or 500 μM hadacidin were added at time 0 as indicated. D[1-14C] thymidine was added after 1/24 h of incubation in the absence of glucose. After a total of 24 h of incubation, cells were harvested, washed, extracted and the intracellular concentration of purine nucleotides were determined as described under Experimental Procedures. All values represent the mean of 3 independent experiments; for sake of clarity the S.D. are not shown but were generally ±15% of the mean.

| Glucose | Myoinosinic Acid | ATP | ADP | GDP |
|---------|------------------|-----|-----|-----|
| (mM)    | and Riboside    | (pmol/10^6 cells) | (pmol/10^6 cells) | (pmol/10^6 cells) |
| 0       | 1.56            | 0.46 | 0.18 |
| 10⁻²    | 1.59            | 0.33 | 0.15 |
| 10⁻³    | 1.61            | 0.35 | 0.27 |
| 10⁻⁴    | 1.73            | 0.33 | 0.16 |
| 10⁻⁵    | 1.84            | 0.43 | 0.19 |
| 10⁻⁶    | 1.93            | 0.43 | 0.27 |
| 10⁻⁷    | 2.00            | 0.43 | 0.27 |

### Table II

**Intracellular Ribose and PRIBSP Concentrations and 114C]Methionine Incorporation into Normal Lymphoblasts**

Normal lymphoblasts were transferred to glucose-deficient media. Variable amounts of glucose and/or 2- deoxyinosinic acid, 2'-deoxyadenosine, and/or 500 μM hadacidin were added at time 0 as indicated. D[1-14C] thymidine was added after 1/24 h of incubation in the absence of glucose. For the determination of the Ribose and PRIBSP concentrations, cells were harvested after a total of 24 h of incubation, washed, extracted and assayed as described under Experimental Procedures. For the determination of 114C]Methionine incorporation, cells were treated for 6 h before the assay was started by the addition of 114C]Methionine and 114C]Alanine as described under Experimental Procedures. All values represent the mean ± S.D. of at least 4 independent experiments, each done in triplicate.

| Ribose Source | Myoinosinic Acid | 114C]Methionine Incorporation |
|---------------|------------------|-------------------------------|
| Glucose       | ATP, ADP, GDP    | pmol/min/10^6 cells          |
| None          | 25.0±15.0        | 4.3±1.0                       |
| 500 μM Glucose| 225±60.5         | 41.7±0.9                      |
| 10 μM Glucose | 61.2±15.1        | 79.7±25.5                     |
| 50 μM 2'-deoxy- | 46.9±11.1        | 72.1±25.7                     |

### Figure 7

**Graph of Cell Volume in PHA-Stimulated Lymphocytes**

The following line graph shows the results of a typical experiment. The following line graph shows the results of a typical experiment. The following line graph shows the results of a typical experiment.

- **A**: The data show a significant increase in cell volume with time. The increase is most pronounced in the presence of PHA and/or 2- deoxyinosinic acid. The addition of 500 μM hadacidin further enhances this effect.
- **B**: The data show a significant increase in cell volume with time. The increase is most pronounced in the presence of PHA and/or 2- deoxyinosinic acid. The addition of 500 μM hadacidin further enhances this effect.
- **C**: The data show a significant increase in cell volume with time. The increase is most pronounced in the presence of PHA and/or 2- deoxyinosinic acid. The addition of 500 μM hadacidin further enhances this effect.

### Table III

**Intracellular Riboflavin and PRIBSP Concentrations and 114C]Methionine Incorporation in Hadacidin-Deficient Lymphoblasts**

Lymphoblasts were transferred to glucose-deficient media. Variable amounts of glucose and/or 2- deoxyinosinic acid, 2'-deoxyadenosine, and/or 500 μM hadacidin were added at time 0 as indicated. D[1-14C] thymidine was added after 1/24 h of incubation in the absence of glucose. Otherwise, the experiment was performed as described in the legend to Table 1.

| Ribose Source | Myoinosinic Acid | 114C]Methionine Incorporation |
|---------------|------------------|-------------------------------|
| Glucose       | ATP, ADP, GDP    | pmol/min/10^6 cells          |
| None          | 36.2±15.7        | 6.2±1.0                       |
| 500 μM Glucose| 235±60.5         | 41.7±0.9                      |
| 10 μM Glucose | 61.2±15.1        | 79.7±25.5                     |
| 50 μM 2'-deoxy- | 46.9±11.1        | 72.1±25.7                     |
Ribose 5-Phosphate and Purine Synthesis

The intracellular RibP and PurP concentrations in PHA-stimulated lymphocytes. The intracellular RibP concentration was determined as described under experimental Procedures. RibP was added at time 0.00. Each point represents the beginning of a 10-wt labelling period. Values represent the mean ± S.D. of at least 5 independent experiments; each experiment was performed in one donor over the entire time course.

Intracellular RibP and PurP Concentrations in PHA-Stimulated Lymphocytes

The intracellular RibP concentration in unstimulated lymphocytes was 19.2 ± 0.7 pmol per cell and did not change during the culture period. Within 2 h after addition of PHA, the concentration increased to 70 ± 2.0 pmol per cell. The RibP concentration continued to increase until 4 h and thereafter remained relatively constant until 24 h. A peak concentration of 339 ± 6.8 pmol per cell was reached at 9 h which was approximately 4.5 times greater than that in the unstimulated controls, equivalent to that in the stimulated controls.

The intracellular PurP concentration in unstimulated lymphocytes was 6.6 ± 1.6 pmol per cell and decreased slightly during the culture period. Within 2 h after addition of PHA, the concentration increased to 10.5 ± 0.8 pmol per cell. The RibP concentration then increased further until 18 h, reached a maximum at 24 h, and then decreased slightly. A peak concentration of 48 ± 2.0 pmol per cell was reached at 9 h, which was approximately 4.5 times greater than that in the unstimulated controls, equivalent to that in the stimulated controls.

Intracellular Concentration of Purine Nucleotides in PHA-Stimulated Lymphocytes

The intracellular concentration of purine nucleotides increased during the first 48 h after the stimulation (Table I). However, the significance of the data was demonstrated by Student's t-test (two-tailed) which was performed in the absence and absence of PHA. The data were expressed as mean ± S.D. The increase in purine nucleotides was followed by a decrease in the concentration of intracellular phosphate and thus the data are evidence of the release of phosphorus from the nucleotides. The data are presented as mean ± S.D. of 72 h.

Intracellular Phosphate Concentration in PHA-Stimulated Lymphocytes

The intracellular concentration of phosphate nucleotides increased during the first 48 h after the stimulation (Table II). However, the significance of the data was demonstrated by Student's t-test (two-tailed) which was performed in the absence and absence of PHA. The data were expressed as mean ± S.D. The increase in purine nucleotides was followed by a decrease in the concentration of intracellular phosphate and thus the data are evidence of the release of phosphorus from the nucleotides. The data are presented as mean ± S.D. of 72 h.

Intracellular Phosphate Concentration in PHA-Stimulated Lymphocytes

The intracellular concentration of phosphate nucleotides increased during the first 48 h after the stimulation (Table II). However, the significance of the data was demonstrated by Student's t-test (two-tailed) which was performed in the absence and absence of PHA. The data were expressed as mean ± S.D. The increase in purine nucleotides was followed by a decrease in the concentration of intracellular phosphate and thus the data are evidence of the release of phosphorus from the nucleotides. The data are presented as mean ± S.D. of 72 h.

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