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Inosine 5′-Monophosphate vs Inosine and Hypoxanthine as Substrates for Purine Salvage in Human Lymphoid Cells[1] (42119)

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Abstract. The ability of inosine 5′-monophosphate vs inosine or hypoxanthine to supply the total purine requirements of mitogen-stimulated human T cells or rapidly dividing human B lymphoblastoid cells was evaluated. Mitogen-stimulated human peripheral blood T cells were treated with aminopterin to inhibit purine synthesis *de novo* and make the cells dependent upon an exogenous purine source. Thymidine was added as a source of pyrimidines. Under these conditions, 25 μM inosine 5′-monophosphate, inosine, and hypoxanthine showed comparable abilities to support [^{3}H]thymidine incorporation into DNA at rates equal to that of untreated control cultures. In parallel experiments with the rapidly dividing human B lymphoblastoid cell line, WI-L2, treatment with aminopterin (plus thymidine) inhibited the growth rate by >95%. The normal growth rate was restored by the addition of 30 μM inosine 5′-monophosphate, inosine, or hypoxanthine to the medium. However, in similar experiments with cell line No. 1254, a derivative of WI-L2 which lacks detectable ecto-5′-nucleotidase activity, only inosine and hypoxanthine (plus thymidine), but not inosine 5′-monophosphate (and thymidine) were able to restore the growth inhibition due to aminopterin. These results show that the catalytic activity of ecto-5′-nucleotidase is sufficient to meet the total purine requirements of mitogen-stimulated human T cells or rapidly dividing human B lymphoblastoid cells and suggest that this enzyme may have functional significance when rates of purine synthesis *de novo* are limited and/or an extracellular source of purine nucleotides is available.

Ecto-5′-nucleotidase (ecto-5′-NT)[3] is an enzyme which is located on the external plasma membrane of subpopulations of human lymphocytes (1). Histochemical studies reveal that 10–25% of adult peripheral T cells and 40–70% of adult peripheral B cells express ecto-5′-NT activity (2–4). This enzyme catalyzes the extracellular dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding ribo- and deoxyribonucleosides (1, 5). These compounds may then be transported inside the cell and reconverted to nucleotides via the purine salvage pathway (see Fig. 1) (6). The contribution of this enzyme toward meeting the total purine requirements of either resting or dividing lymphocytes is, however, unknown. Similarly, it is unknown whether ecto-5′-NT$^+$ and ecto-5′-NT$^-$ lymphocytes differ fundamentally in the route by which they acquire purines for replication and cellular metabolism; i.e., the relative contributions of purine salvage vs. purine synthesis *de novo* in these two cell types are unknown. The experiments described here were designed to determine whether the catalytic activity of ecto-5′-NT is sufficient to provide the total purine requirements of mitogen-stimulated peripheral T cells or rapidly dividing lymphoblastoid cells from exogenous purine nucleotides. Since it is not yet possible to separate lymphocytes according to their ecto-5′-NT phenotype, only mixed populations (ecto-5′-NT$^+$ and ecto-5′-NT$^-$) of T cells could be studied. However, two related human B lymphoblastoid cell lines, one of which is positive (WI-L2) and the other negative (#1254) for ecto-5′-NT activity were also studied and provided pure populations of ecto-5′-NT$^+$ and ecto-5′-NT$^-$ cells. Inosine 5′-monophosphate (IMP)

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[3] Abbreviations used: Ecto-5′-NT, ecto-5′-nucleotidase; IMP, inosine 5′-monophosphate; HxR, inosine; Hx, hypoxanthine; TdR, thymidine; Con A, concanavalin A; PRPP, phosphoribosylpyrophosphate.

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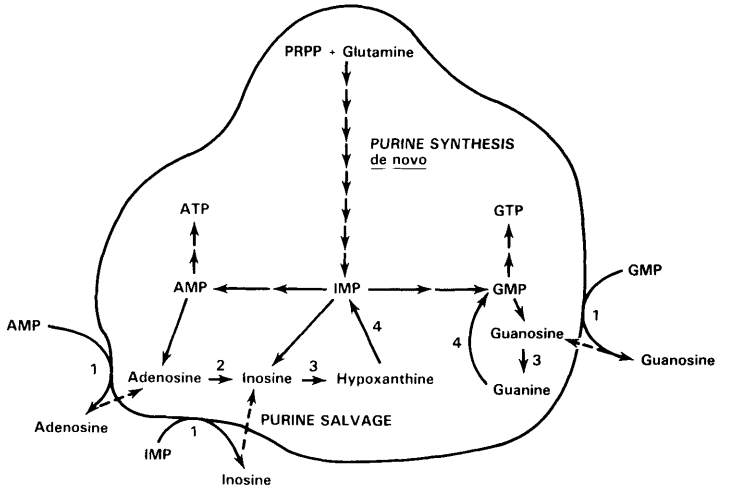

FIG. 1. Cellular purine metabolism. (1) Ecto-5'-nucleotidase, (2) adenosine deaminase, (3) purine nucleoside phosphorylase, (4) hypoxanthine guanine phosphoribosyltransferase.

was chosen as the extracellular purine nucleotide since all other purine nucleotides can be synthesized from it (6).

Materials and Methods. *Isolation of T cells.* Peripheral blood mononuclear cells were isolated from freshly drawn venous blood by Ficoll–Hypaque density gradient centrifugation. T cells were isolated by one cycle of rosetting with neuraminidase-treated sheep red blood cells as previously described (7).

Mitogen stimulation. Six milliliter cultures of T cells were initiated at 1×10^6 cells/ml in 25 cm^2 Corning tissue culture flasks in RPMI 1640 medium with serum-free supplement (HB 101, Hana Biologics, Berkeley, Calif.) with 2 m*M* glutamine, 1 m*M* sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) at 37°C in an humidified atmosphere of 5% CO_2 in air. Serum-free medium was used because fetal bovine serum catalyzes the conversion of IMP to inosine (HxR) and HxR to hypoxanthine (Hx). On Days 3, 4, 5, 6, and 7 after the initiation of the cultures with 0.6 μg concanavalin A/ml (Con A, Sigma, St. Louis, Mo.), and other additions as indicated, quadruplicate 0.2-ml aliquots of each culture were removed, placed in microtiter plates, and pulsed with 1.0 μCi of [^{3}H]thymidine (TdR, 70 Ci/mmole, ICN, Irvine, Calif.) for 4 hr. The cells were harvested onto glass-fiber filters using a multiple automated sample harvester and the filters were counted using scintillation fluid. The results are presented as the means of the quadruplicate samples and represent the cpm of [^{3}H]TdR incorporated in 4 hr by 2×10^5 cells.

Cell lines. Cell lines WI-L2 and No. 1254 were the gifts of Dr. J. Edwin Seegmiller, University of California, San Diego. WI-L2 is a B lymphoblastoid cell line which was established from the spleen of a patient with hereditary spherocytosis (8). The cell line No. 1254 is a spontaneous mutant of WI-L2 which lacks detectable ecto-5'-NT activity; it was isolated fortuitously during routine cloning of WI-L2. Both cell lines were grown in RPMI 1640 with a serum-free supplement as described above. Under these conditions, both cell lines have a doubling time of approximately 24 hr.

Growth rate experiments. Duplicate 5 ml cultures of either WI-L2 or No. 1254 were initiated at 5×10^4 cells/ml with the indicated additions. On each of the next 3 days, duplicate aliquots of each culture were counted with a Coulter counter. The quadruplicate cell counts agreed within 20%; means were used for calculations and plotting the data. Relative growth rates were calculated by

$$\frac{\text{final cell count}}{\text{initial cell count}} - 1.$$

Ecto-5'-NT activity. Ecto-5'-NT activity was determined by measuring the ability of intact

cells to convert [^{14}C]IMP (Amersham, Arlington Heights, Ill., diluted to 6 μCi/μmole with carrier IMP) to [^{14}C]HxR as previously described (7).

Reagents. Aminopterin, TdR, Hx, HxR, IMP, and Con A were purchased from Sigma Chemical Company (St. Louis, Mo.).

Results. *Ecto-5'-NT activity of T cells, WI-L2 and No. 1254.* Ecto-5'-NT activity in isolated T cells was 10.6 nmole/hr/10^6 cells. Ecto-5'-NT activity measured in intact cells from logarithmically growing cultures of WI-L2 and No. 1254 was 18.3 ± 3.5 nmole/hr/10^6 cells for WI-L2 and <0.2 nmole/hr/10^6 cells for No. 1254.

Ability of IMP vs HxR and Hx to support DNA synthesis in mitogen-stimulated T cells treated with aminopterin. T cells stimulated with Con A in purine-free medium showed a maximum rate of [^{3}H]TdR incorporation into DNA 4 days after the initiation of the culture (122,132 ± 5855 cpm/2 × 10^5 cells, Fig. 2). [^{3}H]TdR incorporation was inhibited by 85–95% in mitogen-stimulated T cells treated with 5 μM aminopterin plus 25 μM TdR during the period 3 to 7 days after the addition of Con A. In contrast, the addition

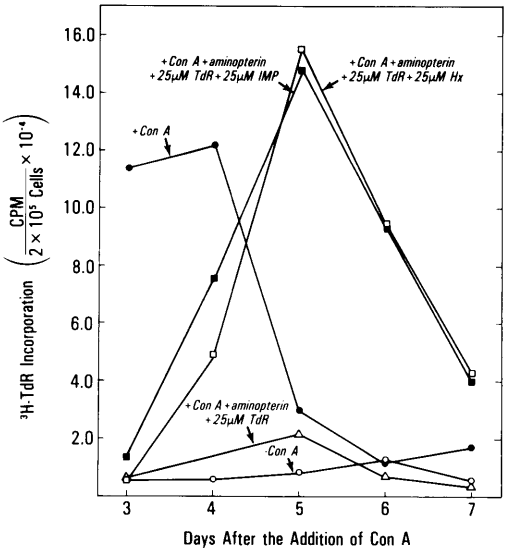

FIG. 2. Ability of hypoxanthine vs IMP to support Con A-induced mitogenesis in aminopterin-treated T cells. ○, −Con A; ●, +Con A; △, Con A + 5 μM aminopterin, 25 μM TdR; □, Con A + 5 μM aminopterin, 25 μM TdR, 25 μM Hx; ■, Con A + 5 μM aminopterin, 25 μM TdR, 25 μM IMP.

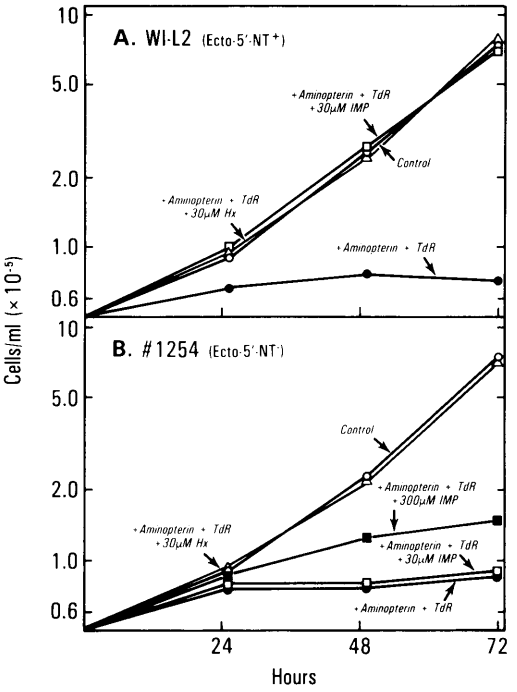

FIG. 3. Growth inhibition of WI-L2 and cell line No. 1254 by aminopterin and reversal by IMP and thymidine. ○, Control cultures, no additions; ●, 5 μM aminopterin + 30 μM thymidine; ▲, 5 μM aminopterin + 30 μM thymidine + 30 μM Hx; □, 5 μM aminopterin + 30 μM thymidine + 30 μM IMP; ■, 5 μM aminopterin + 30 μM thymidine + 300 μM IMP. (A) WI-L2, (B) No. 1254.

of 25 μM TdR plus either 25 μM IMP or 25 μM Hx fully restored the rate of [^{3}H]TdR incorporation in the aminopterin-treated cultures to that of the control cultures (147,989 ± 11,237 cpm/2 × 10^5 cells and 154,939 ± 12,792 cpm/2 × 10^5 cells, respectively) on the fifth day after the addition of Con A. Similar data were found for 25 μM HxR (data not shown).

Inhibition of growth of WI-L2 and No. 1254 by aminopterin and reversal by IMP. Parallel cultures of WI-L2 and No. 1254 were initiated at 5 × 10^4 cells/ml in the presence and absence of 5 μM aminopterin plus 30 μM TdR. The control cultures of WI-L2 and No. 1254 grew at virtually identical rates (Fig. 3). The growth of both cell lines was inhibited by >95% by the addition of aminopterin (plus TdR). The growth of WI-L2 was completely restored to control levels by the addition of 30 μM IMP (plus TdR) to

the culture medium (Fig. 3A), while the addition of the same concentration of IMP (and TdR) had virtually no effect upon the growth rate of No. 1254 in the presence of aminopterin (Fig. 3B). Even 300 μM IMP increased the growth rate of No. 1254 to only 14% of control values. For both WI-L2 and No. 1254, the aminopterin-induced growth inhibition was completely reversed by 30 μM TdR and either 30 μM HxR or 30 μM Hx.

Discussion. Cellular DNA synthesis and proliferation become dependent upon exogenous purines when purine synthesis *de novo* is inhibited by aminopterin. Our results demonstrate that the activity of ecto-5′-NT is sufficient to provide the total purine requirements of cultured human lymphoid cells when IMP is utilized as the exogenous purine nucleotide. In the case of mitogen-stimulated human peripheral blood T cells treated with aminopterin (and TdR to overcome the simultaneous block in pyrimidine biosynthesis), inhibition of DNA synthesis as measured by [^{3}H]TdR incorporation can be overcome equally well by either IMP, HxR, or Hx. The kinetics of the response to Con A are altered, however, with the peak in [^{3}H]TdR incorporation occurring on Day 5 rather than Day 4. Even though only a fraction (10–25%) of human T cells are positive for ecto-5′-NT activity by histochemical stain (2–4), when IMP is the sole purine source, the activity in these cells is sufficient to sustain [^{3}H]TdR incorporation in the entire population at a rate equivalent to that supported by equal concentrations of HxR or Hx. At least two explanations for these results must be considered. First, since only a small fraction of total T cells respond to Con A, these cells may all be ecto-5′-NT$^+$. Alternatively, inosine produced by ecto-5′-NT$^+$ cells might diffuse through the tissue culture medium and act as a purine source for ecto-5′-NT$^-$ cells. Methods must be developed for the separation of ecto-5′-NT$^+$ and ecto-5′-NT$^-$ cells before this issue can be resolved.

Since rates of [^{3}H]TdR incorporation may not reflect true rates of DNA synthesis in cultures treated with TdR and aminopterin, due to alterations in intracellular dTTP pools, a parallel set of experiments was done where mitogen stimulation was monitored by [^{3}H]leucine incorporation into protein. Similar results were obtained; i.e., the aminopterin-induced inhibition of protein synthesis as monitored by [^{3}H]leucine incorporation was completely reversed by the addition of 25 μM TdR plus 25 μM IMP, HxR, or Hx. Therefore, it appears that the ecto-5′-NT activity of human peripheral T cells is sufficient to supply the total purine requirements for proliferation in response to Con A stimulation.

Similar results were obtained when the ability of IMP to supply the total purine requirement needed for proliferation was studied in a pair of rapidly dividing human B lymphoblastoid cell lines, one positive (WI-L2) and the other negative (No. 1254) for ecto-5′-NT activity. In this case, proliferation was measured by cell count. In the case of WI-L2, aminopterin-induced growth inhibition was completely reversed by the addition of 30 μM IMP (plus TdR) to the culture medium. However, in the case of No. 1254, which is negative for ecto-5′-NT activity, 30 μM IMP had virtually no effect upon the growth inhibition caused by aminopterin. Even 300 μM IMP increased the growth rate of No. 1254 to only 14% of control values. This modest increase in growth rate at high IMP concentrations was probably due to the nonenzymatic breakdown of some IMP to HxR or to the low 5′-NT activity present in cell line No. 1254 itself. Since the aminopterin-induced inhibition of growth of No. 1254 can be completely reversed by 30 μM HxR (plus TdR), the failure of 30 μM IMP (plus TdR) to do so must be due to the cells' lack of ecto-5′-NT activity and not to subsequent blocks in the purine salvage pathway.

The results of these experiments show that the catalytic activity of ecto-5′-NT is sufficient to provide the total purine requirements for mitogen-stimulated T cells or rapidly growing B lymphoblastoid cells. Although these experiments do not prove such a function for this enzyme in human T and B cells *in vivo*, they do support the hypothesis that such a function is feasible, at least under specific conditions. Clearly, ecto-5′-NT activity is not required for the growth of cells which have a capacity for purine synthesis *de novo*, as shown by the fact that cell line No. 1254 grows at the same rate as WI-L2 in purine-free media in the absence of aminopterin. However, this enzyme may play a role in

supplying the purines needed for nucleic acid synthesis and cellular metabolism under conditions where purine synthesis *de novo* is limited, such as in resting lymphocytes (9), or under conditions where extracellular nucleotides are available.

Ecto-5'-NT activity increases during the course of human T cell maturation (10). Cortical thymocytes have very low activity (<1.0 nmole/hr/10^6 cells, unpublished results), while medullary thymocytes have an activity similar to that found in adult peripheral T lymphocytes (11). Perhaps the function of ecto-5'-NT in human medullary thymocytes is to salvage purine nucleotides which may be released by the vast numbers of dying cortical thymocytes (12). Ecto-5'-NT activity also increases during the course of human B cell development (13), with adult peripheral B cells having four- to fivefold higher activity than cord blood B cells (27.9 ± 12 vs 5.6 ± 2.8 nmoles/hr/10^6 cells). Whether this increase in ecto-5'-NT activity reflects a shift in the proportion of the total cellular purine requirements met by purine salvage vs purine synthesis *de novo* in ecto-5'-NT^+ vs ecto-5'-NT^- lymphocytes is currently unknown. The establishment of a function for ecto-5'-NT will clarify the significance of the reduced activity of this enzyme found in lymphocytes of patients with a variety of immunodeficiency diseases (14).

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1. Quagliata F, Faig D, Conklyn M, Silber R. Studies on the lymphocyte 5'-nucleotidase in chronic lymphocytic leukemia, infectious mononucleosis, normal subpopulations, and phytohemagglutinin-stimulated cells. Cancer Res **34**:3197–3202, 1974.
2. Matamoros N, Horwitz DA, Newton C, Asherson L, Webster ADB. Histochemical studies for 5'-nucleotidase and alpha-naphthyl (nonspecific) esterase in lymphocytes from patients with primary immunoglobulin deficiencies. Clin Exp Immunol **36**:102–106, 1979.
3. Recker DP, Edwards NL, Fox IH. Histochemical evaluation of lymphocytes in hypogammaglobulinemia. J Lab Clin Med **95**:175–179, 1980.
4. Thompson LF, Saxon A, O'Conner RD, Fox RI. Ecto-5'-nucleotidase activity in human T cell subsets. J Clin Invest **71**:892–899, 1983.
5. Fox IH, Marchant PJ. Purine catabolism in man: Characterization of placental microsomal 5'-nucleotidase. Canad J Biochem **54**:462–469, 1976.
6. Wyngaarden JB, Kelley WN. Gout. In: Stanbury JB, Wyngaarden JB, Fredrickson DS, eds. The Metabolic Basis of Inherited Disease. New York, McGraw–Hill, p916, 1978.
7. Thompson LF, Boss GR, Spiegelberg HL, Jansen IV, O'Conner RD, Waldmann TA, Hamburger RN, Seegmiller JE. Ecto-5'-nucleotidase activity in T and B lymphocytes from normal subjects and patients with congenital X-linked agammaglobulinemia. J Immunol **123**:2475–2478, 1979.
8. Levy JA, Virolainen M, Defendi V. Human lymphoblastoid lines from lymph node and spleen. Cancer **22**:517–524, 1968.
9. Pilz RB, Willis RC, Boss GR. The influence of ribose 5-phosphate availability on purine synthesis of cultured human lymphoblasts and mitogen-stimulated lymphocytes. J Biol Chem **259**:2927–2935, 1984.
10. Edwards NL, Gelfand EW, Burk L, Dosch H-M, Fox IH. Distribution of 5'-nucleotidase in human lymphoid tissues. Proc Natl Acad Sci USA **76**:3474–3476, 1979.
11. Ma DDF, Sylwestrowicz TA, Granger A, Massaia M, Franks R. Janossy G, Hoffbrand AV. Distribution of terminal deoxynucleotidyl transferase and purine degradative and synthetic enzymes in subpopulations of human thymocytes. J Immunol **129**:1430–1435, 1982.
12. Craddock CG, Longmire RL, McMillan R. Production and distribution of lymphocytes and plasma cells. In: Williams WJ, Beutler E, Erslev AJ, Rundles RW, eds. Hematology. New York, McGraw–Hill, p902, 1977.
13. Bastian JF, Ruedi JM, MacPherson GA, Golembesky HE, O'Conner RD, Thompson LF. Lymphocyte ecto-5'-nucleotidase activity in infancy: Increasing activity in peripheral blood B cells precedes their ability to synthesize IgG *in vitro*. J Immunol **132**: 1767–1772, 1984.
14. Webster ADB. Metabolic defects in immunodeficiency diseases. Clin Exp Immunol **49**:1–10, 1982.

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