PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 179, 442–447 (1985)

Purine Nucleoside Phosphorylase Synthesis and Turnover in Human Lymphoid Cells (42121)

KULDEEP NEOTE, EDDIE KWAN, AND FLOYD F. SNYDER

Departments of Medical Biochemistry and Pediatrics, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Abstract. We have studied the turnover and synthesis of purine nucleoside phosphorylase by using a polyclonal rabbit antiserum to this protein. The turnover of purine nucleoside phosphorylase was studied in the B lymphoblast cell, WI-L2, by specific immunoprecipitation of [^{3}H]leucine-labeled proteins. The half-lives for total protein and purine nucleoside phosphorylase were 14.5 and 14.1 hr, respectively. For cells cultured in the presence of inosine the half-life of purine nucleoside phosphorylase was reduced to 11.2 hr. The synthesis of purine nucleoside phosphorylase was analyzed during phytohemagglutinin-stimulated T cell transformation by pulse labeling cells with [^{35}S]methionine. Purine nucleoside phosphorylase synthesis increased greater than 10-fold during the first 12 hr of transformation and continued to a maximum of 30-fold. The relative rate of purine nucleoside phosphorylase labeled to total proteins was 0.04% in unstimulated T cells and increased to 0.18% 12 hr after stimulation. These studies identify some preferential synthesis of purine nucleoside phosphorylase during the early stages of T cell transformation.

The discoveries of the inherited deficiencies of adenosine deaminase (1) and purine nucleoside phosphorylase (2) and their association with severe immunological dysfunction have fostered a variety of studies. Extensive characterization of the metabolism and toxicity of purine nucleosides has been conducted. Purine nucleoside phosphorylase catalyzes the reversible phosphorolysis of the purine ribo- and deoxyribonucleosides, inosine, guanosine, deoxyinosine, and deoxyguanosine. The inherited deficiency of purine nucleoside phosphorylase results in a severe T cell immunodeficiency disease (2). In the present report we describe our findings on the turnover of human purine nucleoside phosphorylase in cultured lymphoblasts and the synthesis of purine nucleoside phosphorylase during phytohemagglutinin-induced T lymphocyte transformation. These studies were accomplished by purification of human erythrocyte purine nucleoside phosphorylase to homogeneity using a previously developed procedure (3) and the preparation of an antiserum to the purified protein.

Materials and Methods. For studies of purine nucleoside phosphorylase turnover, WI-L2 human lymphoblasts were used and cultured as previously described (4). Proteins were labeled by transferring lymphoblasts to leucine-free medium for 3 hr, then passing them into a 75:25 mixture of leucine-free–leucine plus media containing 0.35 mCi/27 ml culture [4,5-^{3}H]leucine, 58 Ci/mmole (I.C.N., Irvine, Calif.). After overnight labeling cells were resuspended in nonradioactive medium and analyzed at various times.

Human T lymphocytes were isolated from peripheral blood by banding on Ficoll–Hypaque and rosetting with 2-aminoethylthiouronium-treated sheep red blood cells (5). Greater than 70% of the T lymphocyte preparation form further rosettes with exposure to sheep red blood cells. T lymphocyte cultures were stimulated with phytohemagglutinin (6) and pulsed at various times for 3 hr with L-[^{35}S]methionine, 1000 Ci/mmole (Amersham, Oakville, Ontario).

Cells were harvested and washed twice with cold media, once with phosphate buffered saline and resuspended in 12 m*M* phosphate buffer at pH 7.0. The cells were lysed by three freeze–thaw cycles in liquid nitrogen and centrifuged at 10,000*g* for 20 min. Supernatants were recovered for immunoprecipitation. Total labeled protein was also analyzed by collection of trichloroacetic acid precipitates on glass-fiber filters.

Samples for immunoprecipitation were made up to 0.5 ml with immunoprecipitation

0037-9727/85 $1.50

buffer (1 mg/ml bovine serum albumin, 5 mg/ml heparin, 10 m*M* dithiothreitol, 0.1% sodium azide in phosphate buffered saline). Previously washed *Staphylococcus aureus* cells (Calbiochem) were added, 0.05 ml, and the mixture incubated at room temperature for 10 min. The *S. aureus* cells were removed by centrifugation for 5 min at 10,000*g* and the procedure was repeated. Purine nucleoside phosphorylase antiserum was added and the mixture incubated at 4°C overnight. The antibody–antigen complex was precipitated by addition of *S. aureus* cells and incubated at room temperature for 10 min. The complex was washed four times with a detergent solution containing 0.15 *M* NaCl, 1% triton X-100, 0.1% sodium dodecyl sulfate (SDS), 10 m*M* Tris base, pH 7.2, 0.1 m*M* leucine or methionine. The final precipitate was taken up in SDS sample buffer for electrophoresis as described. Background and nonspecific immunoprecipitation were analyzed by immunoprecipitating the same lysate a second time. Purine nucleoside phosphorylase antibody immunoprecipitated material and background labeling were analyzed by cutting out the appropriate bands of the dried gel and counting them.

Polyacrylamide slab gel electrophoresis was carried out in 10% gels with 5% stacking gels containing 1% SDS using the discontinuous buffer system (7). Samples were denatured by dissolving in SDS sample buffer (1% β-mercaptoethanol, 1% SDS, and 10% glycerol) and heating at 100°C for 5 min. prior to loading on the gel. Electrophoresis was carried out at 45 mA for 3 to 4 hr after which the gel was fixed overnight, stained with Coomassie brilliant blue, and destained. Fluorography was performed by impregnating the gel with Enhance (New England Nuclear), drying the gel onto Whatman 3 mm and exposing to Kodak XAR-5 film using Dupont intensifying screens at −70°C.

Results and Discussion. Human erythrocyte purine nucleoside phosphorylase was purified to homogeneity as previously described (3) and a polyclonal antibody to this enzyme was produced in the rabbit. We have used this antibody to study the turnover of purine nucleoside phosphorylase in the B lymphoblastoid line WI-L2 and examined the synthesis of this protein during phytohemagglutinin-induced transformation of T cells.

Turnover of purine nucleoside phosphorylase in cultured lymphoblasts. The turnover of purine nucleoside phosphorylase was examined in WI-L2 lymphoblasts in the absence and presence of the substrate inosine and compared to total protein turnover. In studies of thermal inactivation of purine nucleoside phosphorylase in erythrocyte lysates at 70°C, inosine was found to stabilize this activity (8). Lymphoblast proteins were labeled with [^{3}H]leucine, cells were resuspended in nonradioactive medium and aliquots of the culture were removed at various times for measurement of total labeled protein and purine nucleoside phosphorylase. Purine nucleoside phosphorylase turnover was monitored by electrophoresis of immunoprecipitates of the lymphoblast lysates on SDS–polyacrylamide gels. Purine nucleoside phosphorylase from human granulocytes is reported to have a trimeric subunit structure having a subunit molecular weight of 32,800 (9). In Fig. 1 the 32,500 molecular weight subunit of purine nucleoside phosphorylase was identified as a discrete band by fluorography of the dried gel. Adjacent negative lanes show a second immunoprecipitation of each lysate illustrating all of the purine nucleoside phosphorylase was precipitated in the primary exposure to antibody. Appropriate areas were cut out of the gel and counted with the second immunoprecipitation serving as the background for each time point.

A composite plot of total lymphoblast protein and purine nucleoside phosphorylase turnover is shown in Fig. 2. Both total protein and purine nucleoside phosphorylase exhibit essentially the same rate of first order decay having half-lives of 14.5 and 14.1 hr, respectively. These studies indicate the turnover of lymphoblast purine nucleoside phosphorylase closely corresponds to the average of cellular proteins. Of interest, the half-life of purine nucleoside phosphorylase in the presence of a nontoxic concentration of inosine, 0.5 m*M*, was less than in the absence, being reduced to 11.2 hr. While these results are in apparent conflict with the stabilization by inosine in erythrocyte lysates (8), in the intact cell inosine also affects the concentration of another substrate, orthophosphate. The results of

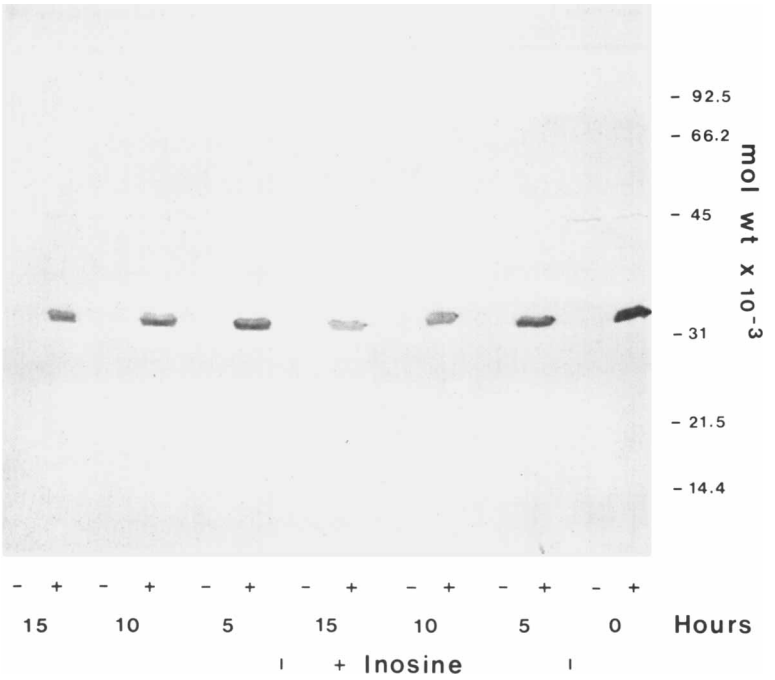

FIG. 1. Turnover of purine nucleoside phosphorylase in human lymphoblast WI-L2 analyzed by specific immunoprecipitation. Proteins were labeled as described with [^{3}H]leucine and cells were resuspended in nonradioactive medium at 2.5×10^5 cells/ml in the absence or presence of 5×10^{-4} *M* inosine. At various times 37.5-ml cultures were harvested for immunoprecipitation with purine nucleoside phosphorylase antibody and determination of total labeled protein. After sodium dodecyl sulfate–polyacrylamide electrophoresis of solubilized immunoprecipitates, fluorography of the dried gel was carried out. Lanes denoted (+) are the primary immunoprecipitates and those denoted (−) are from a second immunoprecipitation of each time point. Molecular weight markers were visualized by staining with Coomassie brilliant blue.

Planet and Fox (10) infer that 0.5 m*M* inosine would reduce intracellular orthophosphate concentrations by as much as twofold in short incubations with intact human erythrocytes. We have shown that purine nucleoside phosphorylase from mouse erythrocyte lysates is stabilized by orthophosphate (11). Thus the role of both orthophosphate and inosine on purine nucleoside phosphorylase stability are interrelated in the cultured cell and need to be further examined.

Synthesis of purine nucleoside phosphorylase during T cell transformation. Lymphocytes of the T cell lineage develop in the microenvironment of the thymus and there is indirect evidence to suggest that purine nucleoside phosphorylase deficiency adversely affects some later stages of T cell differentiation. The purine nucleoside phosphorylase substrate, deoxyguanosine, inhibits lymphocyte proliferation by two mechanisms (12) and has been shown to inhibit proliferation-dependent T cell functions (13). Mitogen-stimulated or proliferating T-cells exhibit increased sensitivity to deoxyguanosine relative to nondividing cells (14, 15). We have therefore examined purine nucleoside phosphorylase synthesis during the transition of human peripheral blood T lymphocytes from the nondividing to mitogen transformed state. Human T lymphocytes were stimulated with phytohemagglutinin and pulsed at various times for 3 hr with [^{35}S]methionine. Lymphocytes were harvested, lysed, and purine nucleoside phosphorylase and total protein synthesis were measured. Fluorography of an SDS–polyacrylamide gel for such an experiment is shown in Fig. 3. As before, adjacent

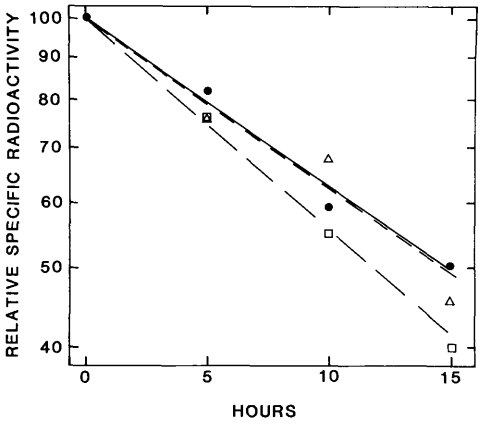

FIG. 2. Purine nucleoside phosphorylase and total protein turnover in human lymphoblast WI-L2. For the experiment described in Fig. 1 the decrease in specific activity relative to the given 0-hr values are plotted for total proteins, 9×10^6 dpm/mg protein (●, solid line); and for purine nucleoside phosphorylase, 3.6×10^3 dpm/mg protein, in the absence (△, broken line) and presence of 5×10^{-4} M inosine (□, broken line).

lanes represent the primary and secondary or reprecipitation of the cellular supernatants with the purine nucleoside phosphorylase antibody. The increase in intensity of label in the 32,500 molecular weight purine nucleoside phosphorylase species is visually apparent over the time course.

A composite plot of total protein and purine nucleoside phosphorylase synthesis during T cell transformation encompasses the results of four experiments (Fig. 4). The maximum rate of label for each individual experiment was set at 100 and other time points were expressed relative to that maximum value. Total protein synthesis increased 10- to 20-fold over the 66-hr time course with a 2- to 5-fold increase in the first 12 hr (Fig. 4B). There was a 30-fold overall increase in the rate of purine nucleoside phosphorylase synthesis and greater than 10-fold increase during the first 12 hr (Fig. 4A). These experiments indicate there is some increased synthesis of purine nucleoside phosphorylase relative to total protein synthesis during T cell transformation.

There is also evidence for some preferential synthesis of purine nucleoside phosphorylase in the early period or first 12 hr after stimulation of T cells. This can best be illustrated by examining the relative rate of purine nucleoside phosphorylase labeling to total protein. At 0 hr the relative rate of purine nucleoside phosphorylase to total protein labeled was 0.04%. By 12 hr this fraction had increased to 0.18% or approximately a fivefold increase and for 40 to 60 hr after mitogen stimulation the fraction remained essentially constant between 0.16 to 0.19%. For comparison the relative amount of purine nucleoside phosphorylase to total protein labeled in continuously dividing WI-L2 B lymphoblasts from Fig. 2 was 0.04%. These findings are similar to estimates of the abundance of purine nucleoside phosphorylase for human

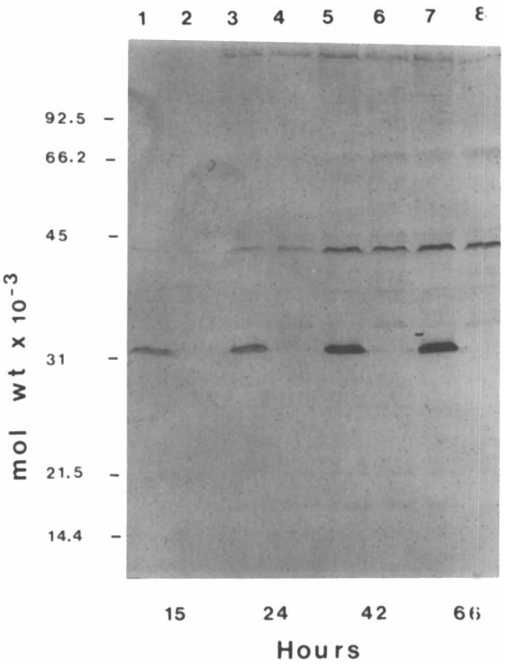

FIG. 3. Immunoprecipitation of purine nucleoside phosphorylase from phytohemagglutinin-stimulated T lymphocytes. T lymphocytes were cultured at a density of 1×10^6 cell/ml and stimulated with phytohemagglutinin. At various times separate cultures were pulsed for 3 hr with [^{35}S]methionine, 0.2 mCi/12 ml culture, after which cells were harvested, lysed, and immunoprecipitated extracts were electrophoresed on a SDS–polyacrylamide gel and examined by fluorography of the dried gel. For each time point odd numbered lanes refer to the primary immunoprecipitation and even numbered lanes the second immunoprecipitation.

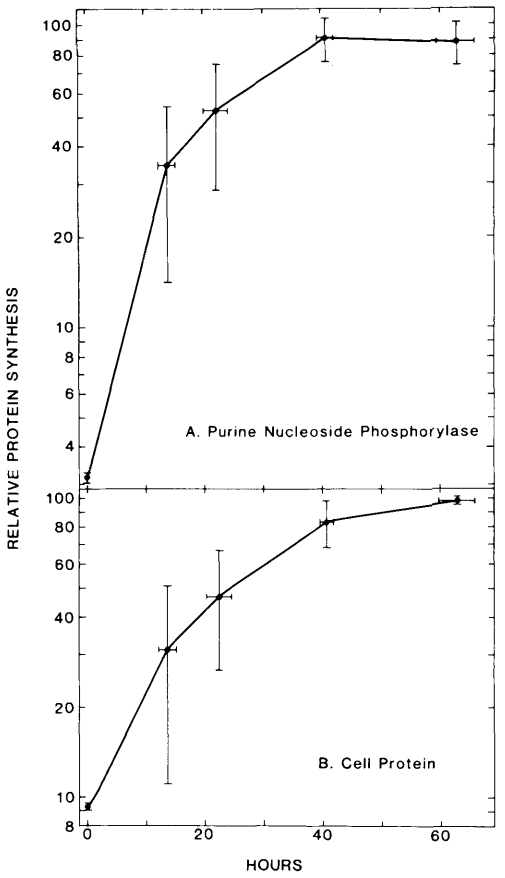

FIG. 4. Changes in the rate of purine nucleoside phosphorylase (A) and total protein synthesis (B) during phytohemagglutinin-induced T lymphocyte transformation. The maximum label from a 3-hr pulse with [^{35}S]methionine for either total protein or purine nucleoside phosphorylase was for each experiment set equal to 100 and other time points were expressed relative to that maximum value. The results of four experiments such as that shown in Fig. 3 were averaged; bars represent standard deviation. Maximum labeling occurred between 40 and 60 hr and was typically for the 3-hr pulse 6–10 $\times$ 10^6 dpm/mg protein for total protein and 1–3 $\times$ 10^3 dpm/mg protein for purine nucleoside phosphorylase.

erythrocytes, 0.04% (3) and for HeLa cells, 0.05% (16). Our studies suggest there is some preferential synthesis of purine nucleoside phosphorylase during the early stages of T lymphocyte transformation but this enzyme remains a relatively low abundance protein even in cells where its function is crucial. As metabolic and pharmacological studies on the role of nucleosides in lymphocyte differentiation, proliferation, and function have already revealed, we will want to know what happens during these processes not only to purine nucleoside phosphorylase but also the other enzymes which metabolize purine ribo- and deoxyribonucleosides.

This work was supported by the Medical Research Council of Canada, Grant MT-6376.

1. Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen AJ. Adenosine deaminase deficiency in two patients with severely impaired cellular immunity. Lancet **2**:1067–1069, 1972.
2. Giblett ER, Ammann AJ, Wara DW, Sandman R, Diamond LK. Nucleoside phosphorylase deficiency in a child with severely defective T cell immunity and normal B-cell immunity. Lancet **1**:1010–1013, 1975.
3. Zannis V, Doyle D, Martin DW Jr. Purification and characterization of human erythrocyte purine nucleoside phosphorylase and its subunits. J Biol Chem **253**:504–510, 1978.
4. Snyder FF, Trafzer RJ, Hershfield MS, Seegmiller JE. Elucidation of aberrant purine metabolism. Application to hypoxanthine-guanine phosphoribosyltransferase- and adenosine kinase-deficient mutants and IMP dehydrogenase and adenosine deaminase inhibited human lymphoblasts. Biochim Biophys Acta **609**:492–501, 1980.
5. Pellegrino MA, Ferrone S, Dierich MP, Reisfeld RA. Enhancement of sheep red blood cell human lymphocyte rosette formation by the sulfhydrylcompound 2-amino ethylisothiouronium bromide. Clin Immunol Immunopathol **3**:324–333, 1975.
6. Snyder FF, Mendelsohn J, Seegmiller JE. Adenosine metabolism in phytohemagglutinin stimulated human lymphocytes. J Clin Invest **58**:654–666, 1976.
7. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685, 1970.
8. Fox IH, Andres CM, Gelfand EW, Biggar D. Purine nucleoside phosphosylase deficiency: Altered kinetic properties of a mutant enzyme. Science (Washington, DC) **197**:1084–1086, 1977.
9. Wiginton DA, Coleman MS, Hutton JJ. Characterization of purine nucleoside phosphorylase from human granulocytes and its metabolism of deoxyribonucleosides. J Biol Chem **255**:6663–6669, 1980.
10. Planet G, Fox IH. Inhibition of phosphoribosylpyrophosphate synthesis by purine nucleosides in human erythrocytes. J Biol Chem **251**:5839–5844, 1976.
11. Snyder FF, Biddle FG, Lukey T, Sparling MJ. Genetic variability of purine nucleoside phosphory-

lase activity in the mouse: Relationship to Np-1 and Np-2. Biochem Genet **21**:323–332, 1983.
12. Spaapan LJM, Rijkers GT, Staal GEJ, Rijksen G, Wadman SK, Stoop JW, Zegers BJM. The effect of deoxyguanosine in human lymphocyte function. I. Analysis of the interference with lymphocyte proliferation in vitro. J Immunol **132**:2311–2317, 1984.
13. Gelfand EW, Lee JJ, Dosch HM. Selective toxicity of purine deoxynucleosides for human lymphocyte growth and function. Proc Natl Acad Sci USA **76**: 1998–2002, 1979.
14. Carson DA, Kaye J, Seegmiller JE. Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: Possible role of nucleoside kinase(s). Proc Natl Acad Sci USA **74**: 5677–5681, 1977.
15. Cohen A, Lee JWW, Dosch HM, Gelfand EW. The expression of deoxyguanosine toxicity in T lymphocytes at different stages of maturation. J Immunol **125**:1578–1582, 1980.
16. Goddard JM, Caput D, Williams SR, Martin DW Jr. Cloning of human purine nucleoside phosphorylase cDNA sequences by complementation in *Escherichia coli*. Proc Natl Acad Sci USA **80**:4281–4285, 1983.

Received January 28, 1985. P.S.E.B.M. 1985, Vol. 179.
Accepted March 6, 1985.