Purine Metabolism in Myeloid Precursor Cells during Maturation

STUDIES WITH THE HL-60 CELL LINE

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ABSTRACT In studies with the human promyelocytic leukemia cell line HL-60, we defined changes in intermediary purine metabolism that appear to contribute to the regulation of terminal maturation in myeloid cells. When HL-60 cells were exposed to compounds that induce maturation, consistent alterations in purine metabolism were found to occur within 24 h of culture. Perturbation of guanosine nucleotide synthesis and decreases of up to 50% in intracellular guanylate pool sizes were associated with the induced maturation of these cells in response to diverse inducing agents. While immature HL-60 cells were observed to synthesize purine nucleotides by both *de novo* and salvage pathways, the activity of both pathways decreased in cells induced to mature, although the relative contribution of purine salvage increased. Moreover, incorporation of the salvage pathway precursor, [^{14}C]hypoxanthine from the intermediate, inosine monophosphate (IMP), into guanylates was reduced by ~65% in induced HL-60 cells, reflecting decreased activity of both hypoxanthine phosphoribosyltransferase and IMP dehydrogenase. When various inhibitors of IMP dehydrogenase (mycophenolic acid, 3-deazaguanosine, and 2-β-D-ribofuranosylthiazole-4-carboxamide) were evaluated for their effects upon HL-60 cells, each agent was found to induce the cells to mature morphologically and functionally. Like other inducers, these agents decreased HL-60 cell proliferation and caused the cells to acquire an ability to phagocytose opsonized yeast and reduce nitroblue tetrazolium. Each agent reduced intracellular guanosine nucleotide pool sizes and induced HL-60 cell maturation at micromolar concentrations. These observations suggest that the size of intracellular guanosine nucleotide pools, the biosynthesis of guanosine nucleotides, and the activity of IMP dehydrogenase may be central to the regulation of terminal maturation in myeloid cells.

INTRODUCTION

Terminal differentiation of eukaryotic cells reflects both a selection of genetic information encoded in DNA and expression of this information in the production of cell-specific proteins and other constituents. Although gene selection appears to be controlled principally by structural and metabolic determinants that are confined to the cell's nucleus and to the chromosomes and nuclear proteins, gene expression may be controlled to a critical degree by the metabolic environment within the cell as a whole. In this study, we have used the human leukemic cell line, HL-60, to examine the role of purine nucleotides (NTD)[1] and their biosynthesis in controlling the terminal differentiation of myeloid cells.

The HL-60 cell line, derived originally from the peripheral blood of a patient with promyelocytic leukemia, can be maintained in continuous culture to provide a renewable source of homogenously immature

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[1] *Abbreviations used in this paper:* AXP, adenylate pools; DMF, dimethylformamide; GMP, guanosine monophosphate; GXP, guanylate pools; HPLC, high-performance liquid chromatography; HPRT, hypoxanthine phosphoribosyltransferase; Hx, hypoxanthine; IMP, inosine monophosphate; IMPD, IMP dehydrogenase; ITS, RPMI 1640 medium supplemented with insulin, transferrin, and selenium; NBT, nitroblue tetrazolium; NTD, nucleotide; PCA, perchloric acid; RA, retinoic acid; XMP, xanthosine monophosphate.

myeloid cells (2). Maturation of these cells is arrested at the early promyelocyte stage. However, it is possible to induce the cells to undergo further differentiation in vitro, and it is this attribute that makes HL-60 cells useful for studying metabolic events that accompany and may contribute to the regulation of myeloid cell maturation (3).

When exposed to various compounds in culture (e.g., dimethyl sulfoxide, dimethyl formamide [DMF], retinoic acid [RA]), HL-60 cells acquire certain morphologic and functional characteristics of mature neutrophils, including an ability to phagocytose and kill microorganisms (2–4) and to respond to chemotactic stimuli (5). These inducible functions characteristic of mature phagocytes are associated with the expression of complement and chemotactic factor receptors and with the development of an activatable oxidase system that can generate H_2O_2 and mediate nitroblue tetrazolium (NBT) reduction (3–6).

Purine NTD metabolism is an appropriate focus for studies of metabolic events that regulate the terminal maturation of myeloid clls. Triphosphorylated purine NTD provide the energy for DNA replication and for RNA transcription and translation (reviewed in references 7 and 8), and they are substrates for protein kinases that regulate these events (reviewed in reference 9). Moreover, there is diverse experimental evidence to suggest a role for purine NTD metabolism in the control of cellular maturation. Studies with the Friend erythroleukemia cell line, for example, have shown that cellular maturation can be promoted by purine bases or purine analogues (hypoxanthine [Hx], 6-thioguanine, and 6-mercaptopurine) which inhibit NTD biosynthesis (10, 11). It has also been observed that lymphoblastic, mono- and myeloblastic leukemia cells express abnormal activity levels of enzymes that regulate the intermediary metabolism of purine NTD, such as adenosine deaminase (12–15), inosine monophosphate dehydrogenase (IMPD) (16), and 5′-methyl thioadenosine phosphorylase (17).

We examined purine NTD metabolism in HL-60 cells both in maintenance culture and under conditions in which the cells were induced to mature. Changes in NTD pool sizes and NTD biosynthesis as well as morphologic and functional changes associated with cellular maturation were evaluated throughout in vitro culture periods. In these studies, we observed that purine salvage is favored as the predominant pathway of NTD biosynthesis in HL-60 cells undergoing maturation. We also observed that certain perturbations in guanylate metabolism are consistent features of induced maturation. Decreases in guanosine NTD pools consistently accompanied maturation; moreover, these changes in NTD pools, which occurred early during induced maturation, could be attributed in part to a discrete inhibition of guanosine NTD biosynthesis. We also determined that inhibitors of IMPD (IMP: NAD-oxidoreductase, EC 1.2.1.14), an enzyme that mediates guanosine NTD biosynthesis from IMP, are active inducers of maturation. Thus, these studies support the concept that the rate of guanosine NTD biosynthesis and the size of guanosine NTD pools have a role in regulating terminal maturation in myeloid cells. Our studies of HL-60 cells also focused attention upon IMPD, an enzyme of intermediary purine metabolism, for the activity of this enzyme appears to be a determinant of the induced maturation of these cells.

METHODS

HL-60 cell line. Continuous suspension cultures of the HL-60 cell line were established in our laboratory with seed cell cultures provided to us by Dr. Joseph Fontana (5) (Department of Medicine, University of West Virginia School of Medicine, Morgantown, WV), who had obtained the cell line earlier from the laboratory of Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD). The cells were maintained in RPMI 1640 medium supplemented either with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Reheis Chemical Co., Phoenix, AZ) or with insulin (5 μg/ml), transferrin (5 μg/ml), and selenium (3 nM) (Sigma Chemical Co., St. Louis, MO). In our studies, serum-free medium supplemented with insulin, transferrin, and selenium is referred to as ITS medium.

Cells were cultured in 75-cm^2 tissue culture flasks (Falcon Plastics, Div. of Becton, Dickinson & Co., Oxnard, CA) and incubated at 37°C in a continuously maintained environment with 7.5% CO_2 in a humidified atmosphere. Cell counts in culture specimens were determined by hemocytometer chamber counting and the viability of cells was assessed by dye exclusion techniques using 0.1% trypan blue (M. A. Bioproducts, Walkersville, MD). Morphology of the cells was evaluated with cytospin preparations (Shandon Southern Instruments, Inc., Sewickley, PA) that had been stained with Wright-Giemsa (Hema-Tek II, Miles Laboratories, Ames Div., Elkhard, IN). For maintenance cultures, cells were seeded into fresh medium (5×10^4 cells/ml) every 4–7 d.

Induced maturation of HL-60 cells. HL-60 cells in log-phase growth were induced to mature by inoculating the cells into fresh media (5×10^4 cells/ml) together with a variety of inducing compounds. Final concentrations of these inducing agents were: RA (Sigma Chemical Co.) 1×10^{-6} to 1×10^{-10} M (18); Hx (Sigma Chemical Co.) 5×10^{-3} M (19); DMF (Eastman Kodak Co., Rochester, NY) 6×10^{-2} M (5). Cell suspensions were incubated in volumes of 50–250 ml for up to 7 d.

The relative maturity of HL-60 cells in culture was evaluated by direct inspection of their morphology and by assessing their ability to phagocytose opsonized yeast and to reduce NBT. For the phagocytosis assay, *Candida albicans* were grown in Sabouraud medium, washed three times in phosphate-buffered saline (PBS), opsonized by incubation with 20% human AB serum in PBS for 2 h at 37°C (4×10^9 organisms/ml), and then stored in aliquots at −20°C. HL-60 cells, washed twice in RPMI 1640 and resuspended at a concentration of 1.0×10^6/ml, were incubated with opsonized *Candida* at a final yeast/cell ratio of 10:1. After incubation for 20 min at 37°C, cell suspensions were fixed onto slides and stained, and the percentage of cells that had ingested

yeast was determined by direct microscopic inspection of at least 200 cells for each experimental condition.

Reduction of NBT by HL-60 cells was determined with a modification of an assay described previously (20). Cells (1×10^6/ml) were incubated for 20 min at 37°C in RPMI 1640 medium with 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate and with 0.1% NBT (both from Sigma Chemical Co.). After incubation, cells were placed onto slides by cytospin, fixed, and stained. The percentage of cells that contained blue-black formazan deposits, the product of NBT reduction, was determined by direct microscopic inspection of at least 200 cells for each experimental condition.

The functional maturity of HL-60 cells, assessed by these techniques, was compared with that of normal human neutrophils separated from venous blood of healthy, adult volunteers by Hypaque-Ficoll/dextran sedimentation techniques (21).

Assessment of purine NTD pools by high-performance liquid chromatography (HPLC). Analysis of purine NTD pools and their biosynthesis in HL-60 cells and in mature neutrophils was achieved by adapting methods described previously (22). Cultured cells were centrifuged at 600 *g* for 10 min and resuspended in 0.5 ml of culture medium. Centrifugation and all subsequent procedures were carried out at 4°C. Perchloric acid (PCA; 1.0 M, 0.5 ml) was added to the concentrated cell suspensions, and after 30 min the acid-insoluble material was removed by centrifugation at 10,000 *g* for 10 min, leaving a clear supernatant that was then neutralized with 10 M KOH. This supernatant, after further centrifugation to remove precipitated salt, was analyzed by HPLC. Extraction of purine nucleosides and NTD was complete after 45 min and the samples were then stored at −80°C. HPLC analysis was performed on samples within 2 wk after preparation. Purine bases and nucleosides present in culture media were analyzed by reverse-phase HPLC on similarly prepared extracts of cell-free supernates.

Cell and media extracts were analyzed for purine NTD, nucleosides, and bases using a Spectra-Physics Inc. (Santa Clara, CA) model 3500B liquid chromatography system equipped with two 740B pumps, a 744 solvent programmer, and a 755 sample injector with a 123-μl loop, and a system I computing integrator. UV absorbance of eluants was measured using an Altex Scientific Inc. (Berkeley, CA) model 152 dual-wavelength detector (254 and 280 nm). For radiotracer studies, radioactivity of eluants was measured simultaneously with a Flo-One (Radiomatic Instruments & Chemical Co. Inc., Addison, IL) radioactive flow detector according to methods described previously from this laboratory (Dr. Webster) (22). Mini-Scint liquid scintillation fluid (Radiomatic Instruments & Chemical Co.) was used throughout these studies.

Purine NTD were chromatographed on an anion-exchange microparticulate column (Partisil-10 SAX, Whatman, Inc., Clifton, NJ). The mobile phase consisted of buffers A (0.01 M potassium phosphate, pH 3.4) and B (0.8 M potassium phosphate, pH 4.3). Buffer A was pumped isocratically for 11 min, after which a linear gradient was established by the addition of buffer B (0–80% B to A over 30 min) at a flow rate of 1.6 ml/min. Purine nucleosides and bases were separated by a reverse-phase method using an Ultrasphere-5 ODS (Altex Scientific Inc.) high-density packed microparticulate column. The mobile phase, consisting of eluant A (0.01 M potassium phosphate, pH 5.6) was pumped isocratically for 3 min, followed by the addition of eluant B (methanol/water, 70:30) in a linear gradient to a final concentration of 40% B to A. Quantification of all compounds was by an external standard method (22).

The effects of changes in cell volume during 7-d culture periods on NTD pool size measurements were evaluated by daily extraction of induced cells with an identical volume of PCA (500 μl). The amount of cell water that contributed to the total extract volume, as a function of changes in cell volume or cell number with time in culture, was determined by adding 10 μl of [8-^{14}C]adenosine (~1,000 dpm) to cell extracts, according to a method described previously (23) in which dilution of a radiolabeled compound is used to access intracellular fluid volume in a cell pellet extract. The concentrations of the isotope in the extracts were then measured. These measurements indicated that differences in total cell water in the extracts did not cause significant changes in the dilution of the nucleoside added to the extracts under culture conditions used in our studies. Therefore, it was concluded that changes in cell volume and cell water with induction made negligible contributions to NTD pool size determinations, and so NTD concentrations were expressed as 10^7 cells.

Assessment of purine NTD biosynthesis. Purine biosynthetic pathways were evaluated in HL-60 cells under different conditions and at different times of culture by incubating cells with radiolabeled precursors, [8-^{14}C]Hx (51.1 mCi/mmol), [8-^{14}C]guanosine (506.0 mCi/mmol), and [^{14}C(U)]glycine (112.0 mCi/mmol) 4 h before extraction and analysis by HPLC with simultaneous UV-radioactivity measurements.

For certain studies, we evaluated HL-60 cell growth, maturation, and NTD biosynthesis with cells that had been cultured in the presence of known inhibitors of the enzyme, IMPD:mycophenolic acid (Eli Lilly & Co., Indianapolis, IN) (24), 3-deazaguanosine (25, 26) and 2-β-D-ribofuranosylthiazole-4-carboxamide (27, 28; kindly provided by Dr. R. K. Robins, Brigham Young University, Provo, UT).

RESULTS

Characteristics of the HL-60 cell line in culture. The HL-60 cell line maintained stable characteristics of morphology, growth, and induced maturation after numerous passages throughout >12 mo of continuous culture in our laboratory. In maintenance culture, the cells retained the morphology of agranular myeloblasts and promyelocytes. When cells were inoculated into fresh media at a concentration of 5×10^4/ml, they consistently exhibited log phase proliferation for 72–96 h, after which cell numbers attained a plateau; cell doubling time during log growth was on the average 22 h in serum supplemented with 10% FBS and 21 h in serum-free ITS media.

As has been reported by others (3–6), HL-60 cells, when cultured in the presence of RA (10^{-6} M), DMF (6×10^{-2} M), or Hx (5×10^{-3} M), acquired various morphologic and functional characteristics of mature neutrophils. During 6 d of culture with each agent, >80% of the cells acquired a capacity to avidly phagocytose opsonized yeast and to reduce NBT. However, the timing with which the cells underwent functional maturation was somewhat different among these inducing agents (Table I). Induced cells also acquired morphologic features of maturity, e.g., a decreased nu-

TABLE I
Functional Maturation of HL-60 Cells

Inducer*	Days in culture: Phagocytosis 1	Phagocytosis 3	Phagocytosis 6	NBT reduction 1	NBT reduction 3	NBT reduction 6
	%			%		
None	17.4±3.0	8.7±2.9	14.9±1.7	10.1±4.6	8.0±6.5	14.7±3.1
DMF, (6.0×10^{-2} M)	34.3±6.3	90.1±3.9	83.1±3.1	38.4±7.2	88.2±6.5	73.2±2.0
A, (1.0×10^{-6} M)	24.2±3.9	83.6±2.1	98.3±1.2	9.4±3.1	87.7±4.3	78.2±1.9
Hx, (5.0×10^{-3} M)	12.0±2.4	38.5±4.3	87.1±3.9	7.1±2.8	25.6±2.8	93.4±1.7

* Cells were cultured in the presence of inducers at concentrations previously determined to result in maximal induction. Percentages of cells containing three or more yeast (*Candida albicans*) or cytoplasmic formazan were assessed by microscopic evaluation of at least 200 cells. Results are expressed as the mean of triplicate samples±SEM.

clear to cytoplasmic ratio, loss of nucleoli, condensation of nuclear chromatin, increased nuclear eccentricity and pleomorphism, and increased cytoplasmic neutrophilia. Morphologic maturation along neutrophilic lines was most evident with RA and DMF, whereas cells exposed to Hx developed a morphology more characteristic of monocytes.

With induced maturation of HL-60 cells, cellular proliferation slowed. As is illustrated in Fig. 1, an inhibition of cell growth was evident after 48 h of culture with each inducing agent and was most pronounced with Hx.

Changes in purine NTD pools during induced maturation of HL-60 cells. Adenosine and guanosine NTD, recoverable from uninduced and induced HL-60 cells during 6 d culture, were quantified by HPLC analysis of perchlorate extracts. As shown in Fig. 2, intracellular pools of adenosine NTD (ADP plus ATP) were highest in uninduced cells after 48 h in culture (265 nmol/10^7 harvested cells), decreasing gradually thereafter. Adenylate levels in cells induced to mature by RA were similar to those of uninduced, control cells. However, adenylate levels in cells exposed to DMF and Hx were decreased during the first 2 d of culture compared with controls.

Unlike the adenylates (AXP), guanosine NTD pools (guanosine triphosphate [GTP] plus guanosine diphosphate [GDP]) in uninduced HL-60 cells (82 nmol/10^7 harvested cells at 24 h of culture) were found to be relatively stable throughout 6 d of culture (Fig. 2). However, in cells induced to mature, there was in all cases a significant decrease in guanosine NTD pools, which was apparent by 24 h of induced maturation and which persisted throughout the 6-d culture periods (Fig. 2). An increased adenylate-to-guanylate ratio (AXP/GXP) was also found to be a consistent characteristic of induced HL-60 cells, and this was primarily a reflection of decreased guanylate levels in the induced cells.

Purine NTD levels in uninduced and induced HL-60 cells expressed as nanomoles/10^7 harvested cells after 72 h of culture are presented in Table II. These data illustrate that both GDP and GTP levels were significantly decreased in HL-60 cells under all conditions of induction, whereas variations of adenylate levels from controls were mixed. These data also show that increased AXP/GXP ratios were a consistent fea-

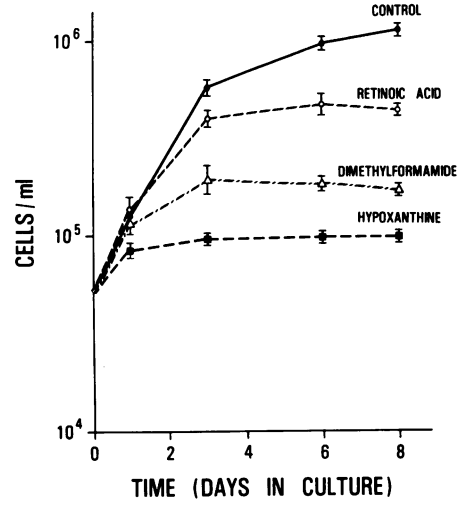

FIGURE 1 Proliferation of HL-60 cells in the absence of inducing compounds (●), and in cultures containing DMF (6×10^{-2} M, △), RA (10^{-6} M, ○), or Hx (5×10^{-3} M, ■). Cells were seeded at an initial concentration of 5×10^4/ml in RPMI 1640 medium with 10% fetal calf serum and incubated under standard conditions. Cell counts were made on the indicated days with a hemocytometer chamber. Results represent mean values from triplicate experiments±SEM.

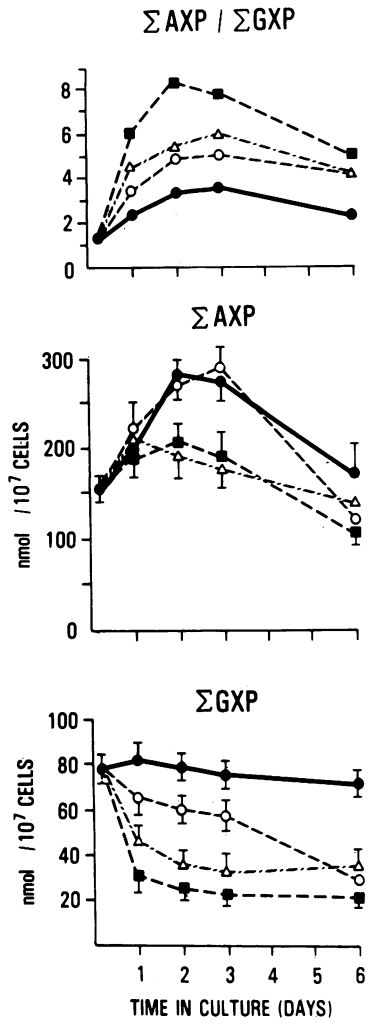

FIGURE 2 Pertubation of guanosine nucleotide pool sizes in HL-60 cells after exposure to DMF (6×10^{-2} M, Δ), RA (10^{-6} M, O), or Hx (5×10^{-3} M, ■), as compared with untreated, control cultures (●). At the indicated times, the cells were processed for purine NTD analyses by extraction with 1 M PCA. Neutralized extracts were injected onto a strong anion exchange HPLC column and absorbance at 254 nm was measured. Results represent mean values from three experiments±SEM.

ture of induction. With or without induction, HL-60 cells maintained triphosphorylated NTD levels that are ~20-fold greater than diphosphorylated NTD levels. The changes in GXP sizes of induced HL-60 cells were not caused or affected by changes in extracellular concentrations of purine bases or nucleosides, since these experiments were carried out with a chemically defined medium that did not contain these compounds. Reverse-phase HPLC of culture media samples taken at various times during culture showed no detectable Hx, guanine, or guanosine.

Purine NTD pools were also measured in normal, mature neutrophils isolated from the blood of healthy adult donors. All NTD levels/10^7 cells were reduced in normal neutrophils compared with HL-60 cells (Table II). Nonetheless, AXP/GXP were significantly higher than those of uninduced HL-60 cells ($P < 0.05$, t test), as was the case with induced HL-60 cells.

Biosynthesis of purine NTD in HL-60 cells. Purine NTD can be synthesized via the *de novo* synthetic pathway by which the amino acid glycine serves as a precursor for the production of inosine monophosphate (IMP), or via salvage pathways by which purine bases are converted to NTD. We therefore carried out studies in which HL-60 cells, harvested at different times and under different conditions of culture, were incubated briefly with [8-^{14}C]Hx and [^{14}C(U)]glycine before extraction and HPLC analysis of NTD. As shown in Table III, [^{14}C]glycine was incorporated into AXP and GXP by both uninduced and induced HL-60 cells, indicating that these leukemic myeloid cells could form NTD via the *de novo* biosynthetic pathway. These cells also actively used [^{14}C]Hx to form NTD by purine salvage.

With the induction of cellular maturation, the relative contribution of the *de novo* pathway for NTD synthesis as indicated by radiolabeled glycine incorporation decreased significantly, although utilization of radiolabeled precursors in general for both salvage and *de novo* pathways decreased with induction (Table IV). While purine salvage (as measured by Hx incorporation) became a relatively more predominant pathway for NTD biosynthesis in induced cells, it was evident at the same time that perturbations in guanylate biosynthesis from the intermediate, IMP, occurred with induction of maturation in HL-60 cells. Incorporation of radiolabel into the GXP was significantly less in induced cells than in uninduced cells (Table III), and this finding indicated that reductions in GDP and GTP pools which characterized HL-60 cell induction could be explained at least in part by an inhibition of guanylate biosynthesis from the intermediate IMP (Fig. 3). Fig. 4 shows purine NTD profiles of uninduced HL-60 cells and of cells induced to mature with RA and DMF at 72 h of culture, together with the relative incorporation of radiolabel from [^{14}C]Hx into the separated NTD. These chromatograms illustrate the relative decrease of incorporation of [^{14}C]Hx into guanylates compared with adenylates in cells induced to mature. For the studies shown, the specific activity (dpm $\times 10^{-2}$/nmol) of the GXP in control cells was 312, whereas the corresponding values for RA- and DMF-induced cells were 75 and 94, respectively.

Conversion of IMP to xanthosine monophosphate (XMP), which is then used to form guanosine monophosphate (GMP), is mediated by the enzyme IMPD. The apparent activity of this enzyme in induced and

TABLE II

*Purine NTD Pool Sizes in Neutrophils and in HL-60 cells 72 h after Induction**

Cell type	Additions	AXP		GXP‡				
		ADP	ATP	GDP	GTP	ΣAXP	ΣGXP	AXP/GXP
HL-60	None	11±2.8	264±26.4	4±1.9	72±6.2	275±27.6	76±9.2	3.5
	RA	10±1.1	277±32.4	2±0.5	56±3.6	287±33.5	58±6.2	5.0
	DMF	8±1.8	174±28.2	2±1.4	29±6.7	182±30.1	31±5.2	5.8
	Hx	8±2.4	177±25.4	2±0.8	22±2.1	185±22.4	24±3.8	7.7
PMN	None	4±1.6	63±7.1	1±0.3	14±4.5	67±6.8	15±3.4	4.5

* Purine NTD pool sizes (nanomoles/10^7 harvested cells) in gradient purified human neutrophils and in HL-60 cells after 72 h in culture with 10^{-6} M RA, 6×10^{-2} M DMF, and 5×10^{-3} M Hx, determined by HPLC on 1 M PCA extracts. Data are the means of three experiments±SEM.

‡ Guanosine NTD pool sizes (GDP, GTP, and GDP + GTP) in HL-60 cells treated with RA, DMF, and Hx were significantly less than those of control, untreated HL-60 cells, $P < 0.001$ (t test).

uninduced HL-60 cells could be estimated from radiotracer studies using [^{14}C]Hx by calculating the incorporation of radiolabel into XMP and guanylates (29). In a similar manner, the apparent activity of the salvage pathway enzyme, hypoxanthine phosphoribosyltransferase (HPRT), which catalyzes the conversion of Hx to IMP could be estimated by summing radiolabel incorporation into adenylates, IMP, XMP, and guanylates (29). Estimates of the relative apparent activities of these enzymes indicated that both were decreased in HL-60 cells induced to mature by RA or by DMF. As shown in Table V, the sum of all derivatives of Hx was decreased by ~70% in induced cells when compared with uninduced cells indicating inhibition of HPRT

TABLE III

*Purine NTD Synthesis by the De Novo and Salvage Pathways in Uninduced and Induced HL-60 Cells**

Treatment	*De novo* synthesis, ([^{14}C]glycine)				Purine salvage synthesis, ([^{14}C]Hx)			
	ADP	ATP	GDP	GTP	ADP	ATP	GDP	GTP
None (control)								
Nanomoles	17	77	5	19	17	77	5	19
Radioactivity (dpm × 10^{-2})	185	670	28	145	538	1645	274	718
Sp act‡	11	9	6	8	32	21	55	38
RA, 10^{-6} M								
Nanomoles	6	71	1	13	6	71	1	13
Radioactivity (dpm × 10^{-2})	6	33	0	2	132	1186	23	190
Sp act	1	<1	0	<1	22	17	23	15
DMF, 6×10^{-2} M								
Nanomoles	6	62	2	10	6	62	2	10
Radioactivity (dpm × 10^{-2})	11	12	2	3	87	716	3	57
Sp act	2	<1	1	<1	15	12	2	6

* HL-60 cells were cultured with RA or DMF for 4 d; [^{14}C(U)]glycine (1.0 μCi/ml) or [8-^{14}C]Hx (1.0 μCi/ml) were added and, after an additional 4 h, NTD were extracted with 1 M PCA and analyzed by HPLC. Values of NTD pool sizes (nanomoles per chromatographic peak) and radioactivity (disintegrations per minute per chromatographic peak) are expressed per 10^7 cells. Results are from a single study and are representative of those obtained in three separate experiments. Data are means of duplicate measurements, which in all cases varied from the mean by <10%.

‡ Sp act = radioactivity (dpm × 10^{-2})/nmol.

TABLE IV

Inhibition of Incorporation of Radiolabeled Precursors into Adenylates and Guanylates in Induced HL-60 Cells after 48 and 96 h°

Treatment	*De novo* synthesis		Purine salvage synthesis	
	Adenylates	Guanylates	Adenylates	Guanylates
	% inhibition			
48 h				
RA	47	94	−0.7	28
DMF	71	83	17	32
96 h				
RA	95	98	40	79
DMF	97	97	63	94

° HL-60 cells, cultured with RA (10^{-6} M) or DMF (6×10^{-2} M) and exposed to radiolabeled precursors of the *de novo* and salvage pathways, were extracted with PCA and analyzed by HPLC (see Table III). Radioactivity in the GXP (GDP plus GTP) and in the AXP (ADP plus ATP) were added and compared with precursor incorporation into purine NTD of control cells. The values are the percentage of inhibition of precursor incorporation in induced compared with uninduced cells and are representative of results obtained in three experiments. Within each experiment, the deviation of duplicate individual measurements from the mean was <10%.

(step 2, Fig. 3). There was also an additional decrease in the levels of guanylate derivatives of IMP, indicating inhibition of IMPD as well (step 1, Fig. 3). In contrast, there was no decrease in the production of adenylate derivatives of IMP that could not be accounted for by the inhibition of IMP production (data not shown).

In related studies, induced and uninduced HL-60 cells were incubated with [8-^{14}C]guanosine (Table VI). These studies showed that guanosine could be used directly by the cells to form guanylates, bypassing bio-

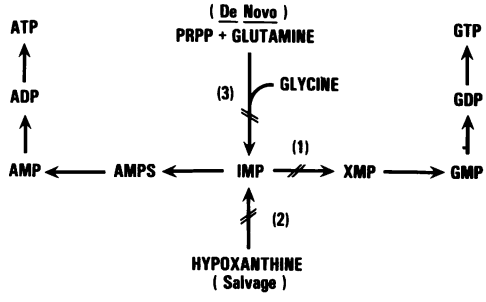

FIGURE 3 *De novo* and salvage pathways for synthesis of purine NTD. Steps of the biosynthetic pathways, (*1*) IMPD, (*2*) HPRT, and (*3*) enzymes of the *de novo* pathway, e.g., phosphoribosylpyrophosphate amidotransferase and ribose-5-phosphate aminotransferase, are indicated. Inhibition of these steps would be expected to result in decreased NTD production in general and decreased guanosine NTD production in particular.

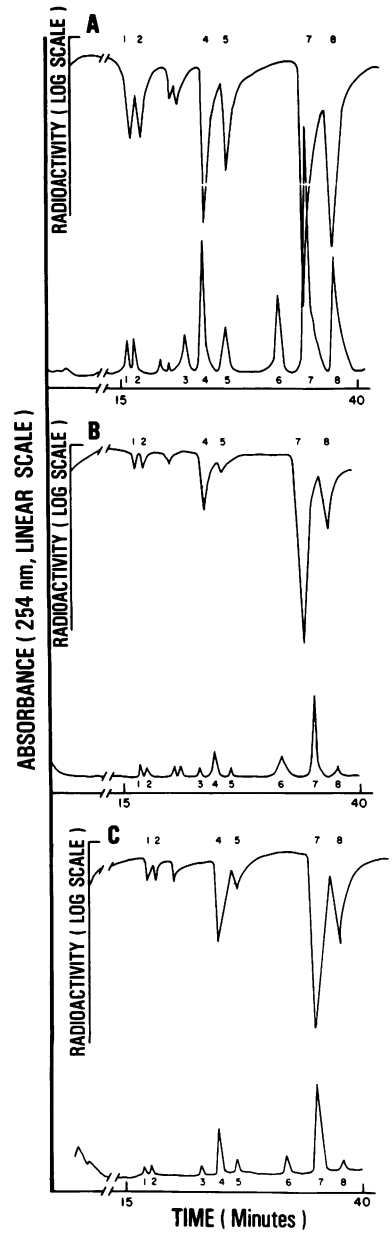

FIGURE 4 Purine NTD profiles of immature HL-60 cells and cells 72 h after induction; (*A*) Profiles of immature HL-60 cells measured by simultaneous UV-adsorption (254 nm)-radioactivity HPLC; (*B*) HL-60 cells 72 h after treatment with RA; (*C*) HL-60 cells after exposure to DMF for 72 h. During the last 4 h of culture, [^{14}C]Hx was added to the cultures, which were then extracted with 1 M PCA as described in Methods. Control cultures contained ~1.5 times as many cells as treated cultures. Peaks were identified by correspondence to standards. Peak numbers: (*1*) GMP, (*2*) XMP, (*3*) uridine diphosphate, (*4*) ADP, (*5*) GDP, (*6*) uridine triphosphate, (*7*) ATP, (*8*) GTP.

synthetic pathways that proceeded via IMP and XMP to GMP. The incorporation of this nucleoside into guanosine NTD was enhanced in the induced cells. In

addition, radiolabel supplied by [^{14}C]guanosine was found in IMP in both induced and uninduced cells, indicating the presence of GMP reductase in HL-60 cells. Moreover, there was again evidence of reduced conversion of IMP to XMP in the induced cells, since the relative recovery of radiolabel in IMP vs. XMP was higher in induced cells than in uninduced cells.

Identification of IMPD inhibitors as inducers of cellular maturation in HL-60 cells. Because studies of purine NTD biosynthesis in HL-60 cells indicated that inhibition of IMPD was a feature of induced cellular maturation, we evaluated the effects of several known inhibitors of this enzyme on HL-60 cells. Cells were incubated with mycophenolic acid (24), 3-deazaguanosine (27), or 2-β-D-ribofuranosylthiazole-4-carboxamide (28) at varying doses for 3–6-d culture periods. Each of these agents inhibited cellular proliferation and induced maturation of the cells. As shown in Table VII and Fig. 5, reduced GXP and the induction of cellular maturation occurred in an equivalent, dose-related fashion. These agents, as expected, also significantly decreased the biosynthesis of guanylates via salvage of Hx (Fig. 6).

Impaired cellular proliferation alone, however, was found not to be associated with the induction of HL-60 cell maturation in the absence of altered GXP. Studies with 3-deazaguanine (26), the base analogue of 3-deazaguanosine demonstrated that this agent was an

TABLE V
Apparent Activities of IMPD and HPRT in Purine NTD Biosynthesis from [^{14}C]Hx in Uninduced and Induced HL-60 Cells

Treatment*	Summation	Apparent activity: Radioactivity	Apparent activity: Inhibition
		dpm/10^7 cells	*%*
IMPD			
None	XMP + GMP	2,534‡	—
RA, 10^{-6} M	+ GDP + GTP	322	87
DMF, 6×10^{-2} M		509	79
HPRT			
None	AMP + ADP + ATP	10,745	—
RA, 10^{-6} M	+ IMP + XMP + GMP	3,246	69
DMF, 6×10^{-2} M	+ GDP + GTP	3,044	71

* HL-60 cells were cultured with RA (10^{-6} M) and DMF (6×10^{-2} M) for 72 h in ITS. During the last 4 h of culture, [^{14}C]Hx (1 μCi/ml) was added. The apparent enzyme activity of IMPD and HPRT were calculated by summing the radioactivity in each of the indicated purine compounds that had been separated by HPLC.
‡ Values are average of duplicate measurements and are representative of results obtained in three separate experiments. Within each experiment, the deviation of individual measurements from the mean was <8%.

TABLE VI
*Incorporation of [^{14}C]Guanosine into Purine NTD of the Guanylate Pathway in HL-60 Cells 72 h after Induction**

Additions	IMP	XMP	ΣGXP‡
None			
Picomoles	ND§	420	2,103
Radioactivity (dpm × 10^{-2})	328	1,103	6,827
Sp act‖	—	263	324
RA, 10^{-6} M			
Picomoles	ND	230	2,053
Radioactivity (dpm × 10^{-2})	778	770	12,343
Sp act	—	334	601
DMF, 6×10^{-2} M			
Picomoles	ND	268	2,182
Radioactivity (dpm × 10^{-2})	1,262	588	11,889
Sp act	—	219	545

* The data are from a single, representative study demonstrating incorporation of [^{14}C]guanosine into purine NTD of HL-60 cells after 72 h in culture with inducing agents. Values of NTD concentration and radioactivity are expressed per 10^7 cells.
‡ ΣGXP is the sum of the values for GMP, GDP, and GTP/10^7 cells.
§ ND, calculation not done; $OD_{254\ nm}$ peak was too small for accurate quantification.
‖ Sp act = (radioactivity/pmol) × 100.

TABLE VII
Influence of IMPD Inhibitors on the Intracellular Purine NTD Concentration of HL-60 Cells

Inhibitor*	Purine NTD concentration‡: GMP	GDP	GTP	ADP	ATP
	% of control				
Mycophenolic acid, 1 μM	0	2	23	58	71
3-Deazaguanosine, 1 μM	5	42	51	83	90
Thiazole nucleoside, 1 μM	23	35	49	102	104

* HL-60 cells were cultured in the presence of mycophenolic acid, 3-deazaguanosine, and thiazole nucleoside at a concentration of 1 μM for 72 h. During the last 4 h of culture, [^{14}C]Hx (1 μCi/ml) was added. Simultaneous UV-radioactivity measurements of HPLC eluates enabled quantification of NTD concentration and radioactivity in neutralized 1 M PCA cell extracts.
‡ Values are means of duplicate measurements and are representative of results obtained in three separate experiments. Within each experiment, the deviation of individual measurements from the mean was <8%.

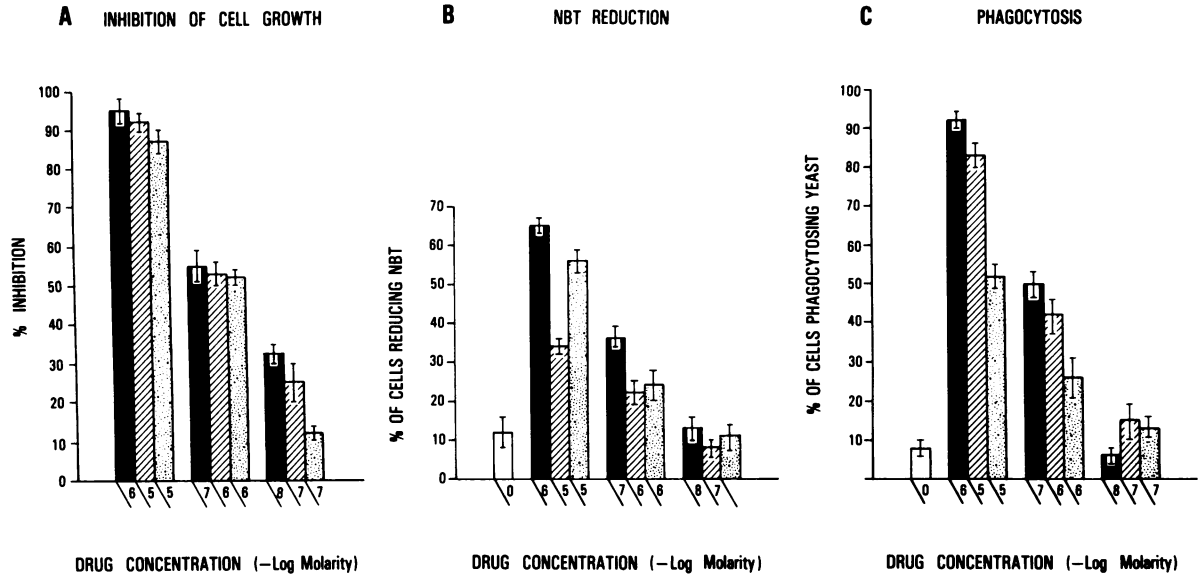

FIGURE 5 Influence of inhibitors of IMPD on HL-60 cell growth and function. The cells were incubated with drugs at the concentrations indicated for 72 h. Percentages of cells reducing NBT and phagocytosing opsonized yeast were evaluated by light microscopy. ■, mycophenolic acid; ▨, 3-deazaguanosine; □, thiazole nucleoside.

inhibitor of HL-60 cell proliferation in culture (>90% at 3 μM), but it did not alter guanosine NTD pools and did not induce maturation (data not shown).

DISCUSSION

Alterations of purine metabolism in immunodeficiency diseases and in malignancy have suggested that purine derivatives have a critical role in regulating the normal growth and maturation of mammalian cells. Our present studies focus upon purine NTD and their biosynthesis in regulating the inducible maturation of a human leukemic myeloid cell line, HL-60.

As observed by others (2–6), this cell line was found to retain consistent characteristics of in vitro growth and inducible maturation throughout many months of maintenance culture. These cells could be shown to undergo morphologic maturation and to acquire the differentiated functions of phagocytosis and NBT reduction in a quantitatively consistent fashion when grown in the presence of known inducers of maturation: DMF, RA, and Hx. When levels of purine NTD present in these cells were measured and the biosynthetic pathways for NTD production analyzed, various changes in NTD pools and NTD synthesis were found to characterize induced maturation.

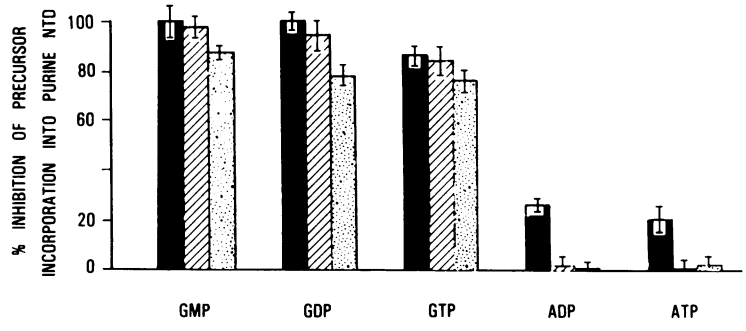

FIGURE 6 Influence of IMPD inhibitors on incorporation of [^{14}C]Hx into purine NTD of HL-60 cells. The cells were cultured with mycophenolic acid (1 μM, ■), 3-deazaguanosine (1 μM, ▨), and thiazole nucleoside (1 μM, □) for 72 h, when [^{14}C]Hx was added for 4 h. Cell extracts were analyzed by UV-radioactivity HPLC.

There was a consistently observed decrease in guanosine NTD pools with cellular maturation. This decrease in guanylates occurred early, before the cells had expressed their differentiated phenotype. On the other hand, no consistent alterations in adenosine NTD pools were found to occur with induced maturation. Primarily because of decreases in guanosine NTD pools, AXP/GXP were greater in induced cells than in immature, uninduced cells, as were the AXP/GXP measured in normal blood neutrophils.

Analysis of NTD biosynthesis in HL-60 cells showed that decreased guanylate pools in induced cells could be accounted for at least in part by an inhibition of NTD production. Both the utilization of radiolabeled glycine to form NTD by the *de novo* pathway and the utilization of radiolabeled Hx to form NTD by the salvage pathway (Fig. 3) were markedly reduced once HL-60 cells were induced to mature. However, the relative inhibition of the *de novo* pathway was greater than that of the salvage pathway. Moreover, the inhibition of guanosine NTD biosynthesis from IMP was greater than that of adenosine NTD biosynthesis.

These studies showed that impaired NTD biosynthesis in cells undergoing induced maturation was associated with decreased production of IMP whether by *de novo* synthesis or by salvage. These studies also indicated that an additional step in the biosynthesis of guanosine NTD was inhibited, i.e., the production of XMP from IMP. Thus, the results suggest that enzymatic steps in the *de novo* pathway and in the salvage of Hx to form IMP (HPRT) were down-regulated in induced cellular maturation. It should be noted, however, that decreased utilization of glycine for purine synthesis by the *de novo* pathway in induced cells could also indicate an increased competition for this amino acid by protein synthetic activity. Nonetheless, it was also evident that induced maturation was associated with an inhibition of IMPD, which mediates the first step in the biosynthesis of guanylates from IMP.

A number of compounds that have attracted interest as antineoplastic agents have been shown recently to be specific inhibitors of IMPD. Three such agents, mycophenolic acid, 3-deazaguanosine, and 2-β-D-ribofuranosylthiazole-4-carboxamide, were evaluated for their effects upon HL-60 cells. Each compound was found to inhibit guanylate biosynthesis via IMP, and to cause a decrease in guanylate pools and an increased AXP/GXP in the cells. Each compound also inhibited cellular proliferation and induced cellular maturation. Considered together, then, these studies indicate that guanosine NTD pool sizes, the production of guanosine NTD, and the activity of IMPD, which mediates guanylate biosynthesis from IMP, can influence the regulation of terminal maturation in myeloid cells.

Decreased intracellular concentrations of guanosine NTD in induced HL-60 cells may be caused in part by an increase in the utilization of guanylates during cellular maturation. It is possible, for example, that there is increased consumption of guanosine NTD for RNA production or to provide energy by hydrolysis of GTP for the burst of protein synthesis that has been observed in induced HL-60 cells (5). However, the finding of decreased NTD biosynthetic activity in cells undergoing maturation and the observation that inhibitors of guanylate production induce maturation clearly suggest that a depletion of guanosine NTD is not simply a consequence of cellular maturation, but rather that it triggers maturation or permits maturation to occur.

The association of reduced GXP and cellular maturation defined by our studies has not been described previously in eukaryotic cells. However, this association has been observed with prokaryotes. In certain species of bacteria, decreases in GTP concentrations have been found not only to accompany but also to induce sporulation (30, 31).

Intracellular concentrations of guanosine NTD could affect cellular maturation by influencing the activity of a variety of regulatory proteins or enzymes. GTP is known, for example, to be a cofactor for adenylate cyclase (32) and for ribosomal proteins that mediate transcription of messenger RNA and the assembly of polypeptides (7–9). In this regard, it is of interest that the product of the *c-src* oncogene of rat-derived murine sarcoma viruses has been characterized as a GTP-binding protein (33). Three cellular oncogenes have been identified in HL-60 cells: *c-myc*, *c-abl*, and *c-ras* (34, 35). The manner in which *onc* gene products may interfere with the normal terminal differentiation of cells is unknown, but their characteristic of GTP binding suggests that this NTD may be required for their activity.

We find it of particular interest that altered guanylate production in induced HL-60 cells appears to involve a down-regulation of IMPD and that inhibitors of this enzyme promote maturation, for alterations in intracellular levels of this enzyme have been associated with malignant transformation. Drugs that inhibit IMPD have also been shown to exhibit significant antitumor effects (reviewed in reference 36). Rat hepatoma cells have been found to have levels of IMPD that are >10 times higher than those of normal liver cells (37). Moreover, studies of normal adult, fetal, and regenerating liver tissue have suggested that there is an inverse relationship between levels of this enzyme and the degree of terminal differentiation of the cells (38). Therefore, our findings support the concept that alterations in the normal regulation of IMPD may interfere with a cell's capacity to undergo terminal maturation and suggest that this enzyme is an appropriate

target for the development of antineoplastic chemotherapy.

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