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Selective Up-Regulation of Type II Inosine 5′-Monophosphate Dehydrogenase Messenger RNA Expression in Human Leukemias[1]

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ABSTRACT

The discovery of isozymes (types I and II) of IMP dehydrogenase (IMPDH; EC 1.1.1.205), the rate-limiting enzyme of *de novo* GTP biosynthesis, has attracted attention as a possible novel approach to cancer diagnosis and selective tumor cell chemotherapy. To elucidate differences in expression and regulation of the two IMPDH isozymes, we examined the steady-state levels of these mRNAs in various types of leukemic cells from patients. Northern blot analysis revealed that type II IMPDH was more active transcriptionally (1.5- to 5.1-fold) in all the leukemic cells examined than in normal lymphocytes, whereas type I expression was similar. The increased expression of type II mRNA in leukemic cells was closely linked with the increase in total IMPDH activity ($r = 0.92$). When leukemic cells from a patient with chronic granulocytic leukemia in blast crisis were separated into blast-rich and mature leukocyte-rich fractions, the expression of type II mRNA correlated positively with the population of immature leukemic cells, whereas type I expression was unchanged. Treatment of leukemic blasts with 12-*O*-tetradecanoyl-phorbol-13-acetate for 5 days resulted in a 90% decrease in the expression of type II mRNA with macrophage-like differentiation, while the expression of type I mRNA was relatively stable. These observations suggest that expression of type II IMPDH is stringently linked with immature characteristics of leukemic cells; thus, it should be a selective target for antileukemic chemotherapy.

INTRODUCTION

Integrated alterations in the activities of key enzymes involved in purine and pyrimidine metabolism have been demonstrated in neoplastic cells (1). In guanine nucleotide synthesis, IMPDH[3] (EC 1.1.1.205) is the rate-limiting enzyme (1, 2) and its activity increases in close connection with cell proliferation (3, 4). Consequently, in neoplastic cells preferential channeling of IMP utilization for guanine nucleotide synthesis takes place (5), resulting in an imbalance in the adenine-guanine nucleotide pools (6, 7). *In vitro* treatment of leukemic cells with an IMPDH inhibitor such as tiazofurin has been shown to inhibit cell proliferation and to induce differentiation by reducing intracellular GTP levels (6, 8–11). In clinical studies, tiazofurin provided hematological remissions in patients with refractory leukemias (12–14). Thus, IMPDH is an important regulatory enzyme for guanylate biosynthesis and also a target for antileukemic chemotherapy.

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[3] The abbreviations used are: IMPDH, IMP dehydrogenase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AML, acute myelogenous leukemia; CGL, chronic granulocytic leukemia; CGL-CP, CGL in chronic phase; CGL-BC, CGL in blast crisis; CLL, chronic lymphocytic leukemia; NBT, nitroblue tetrazolium; SDS, sodium dodecyl sulfate; SSPE, a phosphate-buffered salt solution containing 0.18 M NaCl-10 mM sodium phosphate, pH 7.7-1 mM EDTA; cDNA, complementary DNA.

Human IMPDH cDNA was first cloned by Collart and Huberman (15) and the alteration in expression of this gene was examined in leukemic cells during differentiation (11, 16). Recently, Natsumeda *et al.* (17, 18) isolated two distinct IMPDH cDNAs (types I and II) from a human spleen cDNA library and demonstrated that in normal leukocytes type I IMPDH was transcriptionally more active than type II, whereas in ovarian tumor cells type II predominated. Sequence analysis of these two genes revealed that both cDNAs encode closely related proteins with 84% sequence identity. We constructed expression plasmids with these cDNAs and demonstrated that both proteins possess IMPDH activities (18). Thus, in human cells IMPDH appears to consist of two distinct isozymes which might be differentially regulated. The present study was conducted to elucidate: (*a*) the steady-state expression of type I and II mRNAs in various leukemic cells freshly isolated from patients and to compare it with that in normal hematopoietic cells, (*b*) the alteration in expression of type I and II mRNAs during differentiation induced in fresh leukemic cells, and (*c*) the genomic structures of type I and II regions in leukemic cells.

MATERIALS AND METHODS

Patients. The nature of the procedures was explained fully to the patients and informed consent statements were obtained in a manner consistent with the regulations of the Institutional Review Board and Subcommittee Reviews for Human Subjects Research at Indiana University School of Medicine. The characteristics of the 10 patients with leukemia examined in this study are shown in Table 1. Two patients had AML, 5 patients had CGL, and 3 had CLL.

Cell Separation. Peripheral blood cells were obtained from patients before the initiation of chemotherapy and from healthy adult volunteers. Mononuclear cells were isolated by the standard Ficoll-Hypaque method (19). For isolation of blast-rich and mature leukocyte-rich fractions, peripheral blood was processed as reported previously (20).

Assay of IMPDH Activity. IMPDH activity was assayed as described before (21). Protein concentration was determined by a protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

Cell Growth and Maturation. Viable cell counts were determined by the trypan blue dye exclusion method. Cytocentrifuge preparations were made and May-Grünwald-Giemsa staining was performed. At least 200 cells were counted for morphological analysis. Functional maturation was assessed by the ability to reduce NBT because this capacity is present only in the differentiated cells (22).

Adherence to Plastic Surface. Adherence was examined by plating 1×10^6 cells/ml of medium in 25-ml Falcon tissue culture flasks (Becton Dickinson Co., Lincoln Park, NJ). After incubation for 1–5 days at 37°C, nonadherent cells were harvested and adherent cells were removed by 0.25% trypsin (GIBCO, Grand Island, NY) and counted separately.

RNA Preparation and Northern Blot Analysis. Total cellular RNA was isolated according to the method reported previously (23). Total RNA (10–20 μg) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane (Hybond N; Amersham, Arlington, IL), and hybridized with ^{32}P-labeled probes. The hybridizations were carried out for 16–24 h at 42°C in 50% (v/v)

Table 1 *Characteristics of the patients*

Case	Diagnosis	WBC count (×10⁹/liter)	WBC differential (%)				Total IMPDH activity (nmol/h/mg protein)	IMPDH mRNA expression[a]	
			Blasts[b]	Mature[b]	Lymph[b]	Others		Type I	Type II
	Normal lymphocytes[c]						8.7 ± 1.3	1.0	1.0
1	AML	10.5	88	0	11	1	52.7	0.9	3.9
2	AML	77.4	77	8	13	2	55.6	1.1	5.1
3	CLL	54.6	0	23	76	1	46.2	0.8	3.4
4	CLL	22.0	0	17	83	0	41.2	0.7	2.2
5	CLL	23.0	0	15	84	1	38.1	0.9	2.2
6	CGL-CP	158.8	8	50	12	30	15.7	0.9	1.5
7	CGL-BC	184.0	39	40	4	17	33.6	0.9	2.3
8	CGL-BC	36.2	79	14	7	0	62.2	1.0	5.1
9	CGL-BC	114.0	52	32	3	13	59.9	ND[d]	ND
10	CGL-BC	684.0	71	15	4	10	68.3	ND	ND

[a] Expression of type I and II mRNA documented by Northern blot (Fig. 1). After standardization by corresponding γ-actin mRNA, they were expressed relative to the level of those in normal lymphocytes examined under the same analytical conditions, taken as 1.0.
[b] Blasts, blasts + promyelocytes + myelocytes; Mature, metamyelocytes + neutrophils; Lymph, lymphocytes.
[c] Mean ± SE of 3 healthy volunteers.
[d] ND, not determined.

formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% (w/v) SDS, and 200 μg/ml denatured salmon sperm DNA. After hybridization, membranes were washed consecutively with solutions of 2 × SSPE/0.1% SDS at room temperature; 1 × SSPE/0.1% SDS at 65°C; and finally twice with 0.1 × SSPE/0.1% SDS at 65°C and then exposed to Kodak X-Omat XAR films (Rochester, NY) using an intensifying screen for 12–72 h. Blots were quantitatively analyzed by a densitometer (Bio-Rad, Model 620) and levels of each expression were standardized by the expression of the corresponding γ-actin gene in order to account for possible differences in sample loading.

Isolation of High Molecular Weight DNA and Southern Blot Analysis. High molecular weight DNA was isolated by an SDS/proteinase K method (24). DNA (5 μg) was digested to completion with the indicated restriction enzymes according to the directions of the manufacturer. Digested DNA was fractionated by electrophoresis and transferred to a nylon membrane. The conditions of hybridization solution and autoradiography were the same as for Northern blot analysis.

DNA Probes. Probes used in this study were a *Bam*HI/*Eco*RI fragment of IMPDH-I cDNA (1352–2258 base pairs) (17) and a *Pst*I/*Eco*RI fragment of IMPDH-II cDNA (606–1646 base pairs) (17). Both probes contained a part of the coding region and 3′-noncoding region of the respective cDNAs. For Northern blot analysis, control hybridizations were performed with a human γ-actin cDNA (25). All probes were labeled by random priming (26) to specific activities of 1 × 10⁹ cpm/μg.

RESULTS AND DISCUSSION

Expression of Type I and II mRNAs in Fresh Leukemic Cells. To elucidate the possible difference in expression of type I and II IMPDHs, we first examined the steady-state levels of these mRNAs in normal lymphocytes and in various types of leukemic cells from patients. The characteristics of the patients and total IMPDH activities in the leukemic cells are listed in Table 1. Total IMPDH activities in normal lymphocytes were 8.7 ± 1.3 nmol/h/mg protein (mean ± SE), and in leukemic cells they were elevated 1.8- to 7.8-fold. The higher IMPDH activities in leukemic cells compared to normal lymphocytes are consistent with previous reports (12, 27, 28). Northern blot analysis demonstrated that the expression of type I mRNA was similar in lymphocytes and leukemic cells, whereas the expression of type II mRNA was higher in all the leukemic cells than in normal lymphocytes examined under the same analytical conditions (Fig. 1). Densitometric analysis of the blots revealed that the expression of type II transcripts was 1.5- to 5.1-fold higher in leukemic cells than in normal lymphocytes (Table 1). Among leukemic cells, type II mRNA expression was much

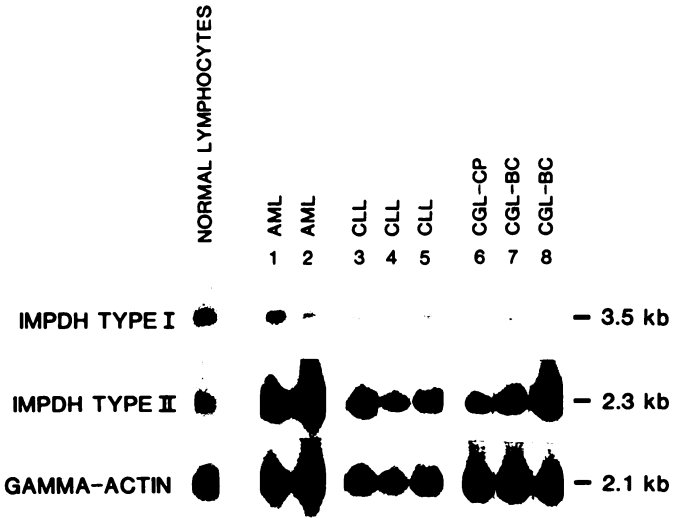

Fig. 1. Expression of type I and II IMPDH mRNAs in fresh leukemic cells and normal lymphocytes. Total RNAs (20 μg) were electrophoresed, transferred to a nylon membrane, and sequentially hybridized with type I and type II and γ-actin probes as described in "Materials and Methods." *kb*, kilobases.

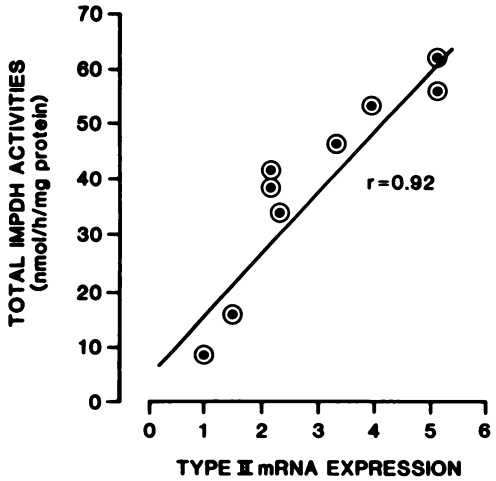

Fig. 2. Comparison of type II mRNA expression with total IMPDH activities. Type II mRNA expression was calculated as the level relative to that in normal lymphocytes after standardization by the corresponding γ-actin expression (Table 1).

Table 2 *Characteristics of fractionated CGL-BC cells*

CGL-BC cells from Case 9 were layered over a discontinuous gradient of Hypaque-dextran (20) and centrifuged at 400 × *g* for 12 min. After centrifugation, leukemic cells at interphase were designated as blast-rich fraction and those at the bottom as mature leukocyte-rich fraction.

Fractions	WBC differential (%)				Total IMPDH activity[a]	IMPDH mRNA expression[b]	
	Blast[c]	Mature[d]	Lymphocytes	Others		Type I	Type II
Unfractionated	52[e]	32	3	13	59.9 (100)	1.0	1.0
Blast-rich	85	0	12	3	93.2 (155)	0.9	1.7
Mature leukocyte-rich	8	71	1	20	23.8 (40)	1.1	0.3

[a] nmol/h/mg protein. Numbers in parentheses, percentages of unfractionated cells.
[b] Expression of type I and II mRNA was examined by Northern blots. After standardization by corresponding γ-actin mRNA, type I and II mRNA in blast-rich and in mature leukocyte-rich cell fractions were expressed relative to the level of those in unfractionated cells.
[c] Blasts + promyelocytes + myelocytes.
[d] Metamyelocytes + neutrophils.
[e] Percentage of cells stained with May-Grünwald-Giemsa.

higher in AML cells (Cases 1 and 2) than in CLL cells (Cases 3–5). In CGL (Cases 6–8), leukemic cells with the larger population of immature leukemic cells exhibited the higher expression of type II mRNA. The expression of type II mRNA in leukemic cells correlated significantly with the total IMPDH activities in the cells ($r = 0.92$) (Fig. 2). These observations suggest that the increase in total IMPDH activity in leukemic cells was largely due to an increase in type II isozyme.

Expression of Type I and II mRNAs in Leukemic Cells at Different Maturation Stages. In contrast to leukemic cells, type I mRNA is the dominant species in peripheral leukocytes (17). In order to clarify the relationship between expression of IMPDH isozymes and cell maturation, we examined the mRNA levels of both IMPDHs in leukemic cells derived from the same clone at different maturation stages. Because CGL is a disease of monoclonal origin, we fractionated leukocytes from a patient with CGL-BC (Case 9) using a discontinuous gradient of Hypaque-dextran (20). In unfractionated leukocytes, 52% of the cells were morphologically classified as immature leukemic cells (blast + promyelocyte + myelocyte) and 32% were mature leukemic cells (metamyelocyte + neutrophil) (Table 2). Total IMPDH activity in these cells was 59.9 nmol/h/mg protein. After centrifugation at 400 × *g* for 12 min, leukemic cells at the interphase fraction mainly consisted of immature leukemic cells (85% population in this fraction) and showed IMPDH activity of 93.2 nmol/h/mg protein (blast-rich fraction). By contrast, the cell pellet mainly included mature leukemic cells (71% population) with a relatively lower IMPDH activity of 23.8 nmol/h/mg protein (mature leukocyte-rich fraction). Northern blot demonstrated that type II mRNA expression was 1.7-fold higher in the blast-rich fraction and 70% lower in the mature leukocyte-rich fraction than in unfractionated cells, but type I mRNA expression was similar in all cell fractions (Table 2). Thus, the expression of type II mRNA decreased along with cell maturation, while that of type I was constitutive. The relatively low expression of type II mRNA in leukemic cells with CGL-CP (Case 6) might be due to the high population of differentiated leukemic cells.

Alteration in Expression of Type I and II mRNAs in Leukemic Cells during Differentiation. To obtain further evidence for the selective regulation of type I and II isozymes, we examined the alteration in expression of these mRNAs in leukemic cells

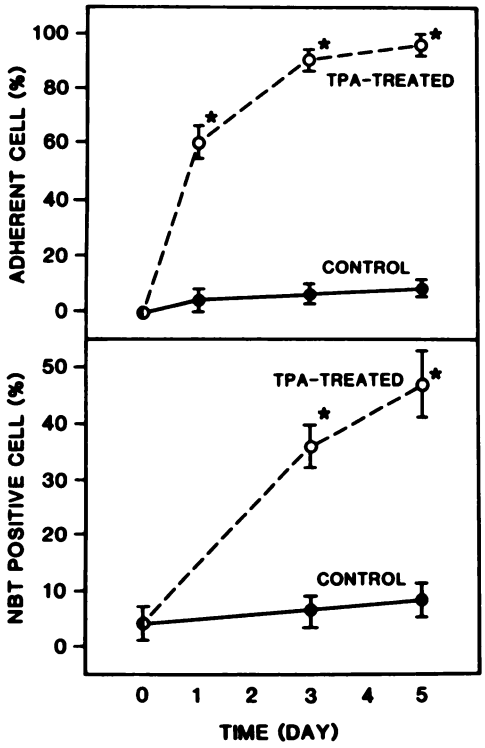

Fig. 3. Effect of TPA on differentiation in leukemic blasts. Leukemic blasts were isolated from Case 10 and incubated in the presence or absence of TPA (0.1 μM). Adherent cells and NBT-positive cells were counted as described in "Materials and Methods." *Points*, means (*bars*, ±SE) of triplicate samples. *Asterisks*, significantly different from control cells ($P < 0.05$).

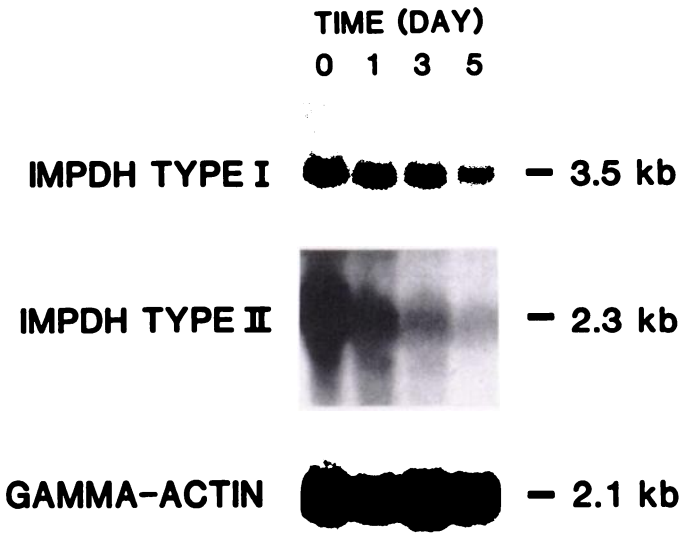

Fig. 4. Effect of TPA on type I and II mRNA expression in leukemic blasts. Mononuclear cells were isolated from Case 10, and the cells were treated with TPA (0.1 μM) at the times indicated. Total RNAs (20 μg) were electrophoresed, transferred to a nylon membrane, and sequentially hybridized with type I and II IMPDH and γ-actin probes. kb, kilobases.

during differentiation induced by TPA. Leukemic blasts were isolated (22) from a patient with CGL-BC (Case 10) and incubated with TPA in RPMI 1640 medium containing 15% fetal bovine serum. Twenty-four h after incubation 60% of the viable cells became adherent to the flasks (Fig. 3). Five days after incubation, 96% of the viable cells were adherent with a macrophage-like appearance and 47% of the viable cells showed NBT-reducing activity. Northern blot analysis showed that expression of type II mRNA decreased to <10% of the pretreatment level during the 5-day incubation, but that of type I mRNA remained >70% of the initial level (Fig. 4). The rapid decrease in type II transcript during the TPA-induced differentiation is consistent with previous reports (11, 16). The evidence as a whole indicates the important role of the type II IMPDH isozyme in the regulation of cell proliferation and differentiation.

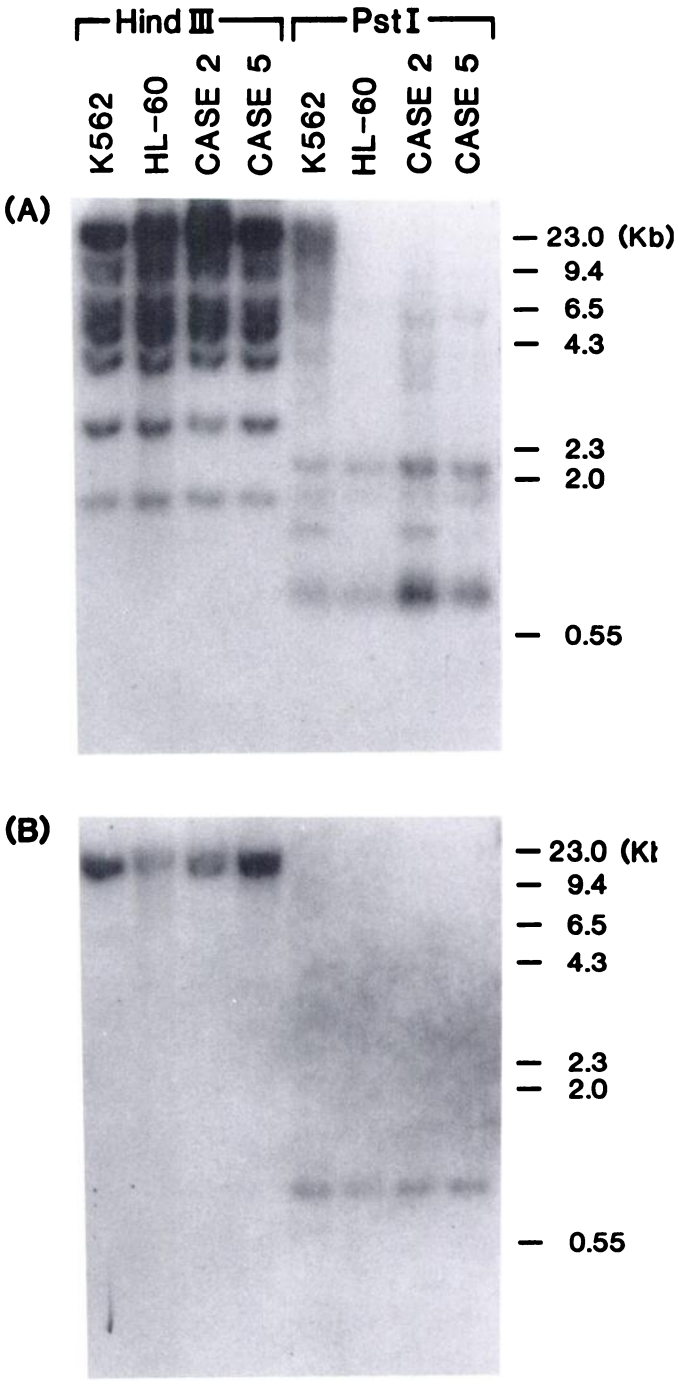

Fig. 5. Southern blot analysis of genomic DNA from leukemic cells probed with type I and type II IMPDH cDNAs. High molecular weight DNA was isolated from HL-60, K562, and fresh leukemic cells (Cases 2 and 5). DNA (5 μg) was digested with *Hind*III or *Pst*I, electrophoresed, transferred to a nylon membrane, and hybridized with (*A*) type I and (*B*) type II IMPDH probes. *Ordinates*, positions of size marker in kilobases (*Kb*).

DNA Structure of Type I and II IMPDH Genes in Leukemic Cells. To elucidate possible structural changes in type I and II IMPDH locus in leukemic cells, Southern blot analysis was conducted in two leukemic cell lines (HL-60, K562) and two fresh leukemic cell populations (Cases 2 and 5) which had high IMPDH activities. The representative blots show that no macroscale gene alterations, such as amplification or rearrangement, were detected in the two IMPDH genes in these cells (Fig. 5).

Biological Roles of the IMPDH Isozymes. Guanine nucleotides play an important role not only as a precursor of nucleic acid biosynthesis but also as mediators of signal transduction (29). Since IMPDH is a key enzyme in regulating cellular GTP levels, alteration in expression of this enzyme might be involved in many biological events. The discovery of isozymes of IMPDH and subsequent studies revealed that human IMPDH consists of two distinct isozymes which are differentially distributed (17, 18).

Although the biological roles of the respective isozymes remain unclear, the constitutive expression of type I isozyme (shown above) may indicate its "housekeeping" function. On the other hand, the expression of type II mRNA is closely linked with the proliferative state of the cells; high expression was observed in all examined leukemic cells, especially in the cases with AML and CGL-BC which had a rapid clinical course.

The selective increase in type II mRNA in leukemic cells might provide advantages for leukemic cell-specific chemotherapy. One of the serious problems associated with antileukemic chemotherapy is that most of the known agents are not specific enough for leukemic cells. As a consequence, some normal cells are killed during treatment, causing morbidity and at times death of the host. The development of analogues which selectively inhibit type II IMPDH activity, but not type I, might overcome these side effects of conventional agents. Therefore, the possible differences in properties between the two IMPDH isozymes is of interest and further studies are under way.

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