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Synthesis of adenine and guanine nucleotides at the 'inosinic branch point' in lymphocytes of leukemia patients

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Abstract

The synthesis of purine nucleotides has been studied in human peripheral blood lymphocytes from healthy subjects and patients affected by B-cell chronic lymphocytic leukemia (B-CLL). The rate of the synthesis was measured by following the incorporation of ^{14}C-formate into the nucleotides of lymphocyte suspensions. The whole sequence AMP → ADP → ATP was found reduced in B-CLL lymphocytes; in the case of guanylates only the last step of the sequence GMP → GDP → GTP was significantly lower in the same cells. From the analysis of these results, combined with previous data, we conclude that purine metabolism undergoes an imbalancement during CLL, which is partially compensated, and point out the importance of studying concomitantly purine metabolism and nucleic acid synthesis in leukemia cells.

Keywords: Purine nucleotide; B-cell chronic lymphocytic leukemia; Nucleotide synthesis

1. Introduction

In a previous paper, we studied the purine nucleotide metabolism in lymphocytes of healthy subjects and B-cell chronic lymphocytic leukemia patients [1]. We found a substantial decrease in absolute nucleotide content, except for NAD and IMP. This decrease was less striking when values were expressed per unit of proteins.

The activities of many enzymes of nucleotide metabolism were investigated in lymphocytes from both subjects: the enzymes involved in nucleotide catabolism (AMP deaminase, purine nucleoside phosphorylase, adenosine deaminase), especially 5′-nucleotidase, had reduced activities in leukemia patients. As far as the two enzymes involved in the 'salvage pathway' are concerned, hypoxanthine-guanine phosphoribosyltransferase (HGPRT) was decreased, and adenine phosphoribosyltransferase (APRT) showed slightly increased activity. In preliminary experiments, we also followed the incorporation of ^{14}C-formate into IMP, total adenine nucleotides and total guanine nucleotides. We found very low labeling, especially in total adenylates of B-CLL lymphocytes, and we concluded that nucleotide synthesis was impaired. All these changes indicate an imbalance in purine metabolism in B-CLL; they are also

Abbreviations: APRT, adenine phosphoribosyltransferase; B-CLL, B-cell chronic lymphocytic leukemia; FACS, fluorescence-activated cell sorter; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline

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potential markers of lymphoid malignancies and may be useful in chemotherapy.

The intracellular nucleotide pool is synthesized by interdependent metabolic processes: (1) 'de novo synthesis' produces the purine ring from non-purine precursors. It includes several steps and leads to the formation of IMP from PRPP [2]; (2) 'the inosinic branch point' is the section of the sequence where IMP is channeled into AMP and GMP. AMP-succinate synthetase is involved in the formation of AMP-succinate, which is split into AMP+fumaric acid by AMP-succinate lyase. IMP-dehydrogenase and GMP-synthetase form first XMP and then finally GMP, from IMP; (3) the salvage pathway can readily form mononucleotides from purine bases and their nucleosides. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) catalyzes the formation of IMP from hypoxanthine and GMP from guanine; this enzyme returns the major products of purine nucleotide catabolism to nucleotide forms. Adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) catalyzes the conversion of adenine to AMP, by a reaction similar to that of HGPRT: it plays a minor role in purine salvage since adenine is not a significant product of purine nucleotide catabolism [3]; and (4) different reactions produce nucleoside di- and triphosphates from AMP and GMP. AMP is transformed into ADP by myokinase (EC 2.7.4.3):

$$ATP + AMP \leftrightarrow ADP.$$

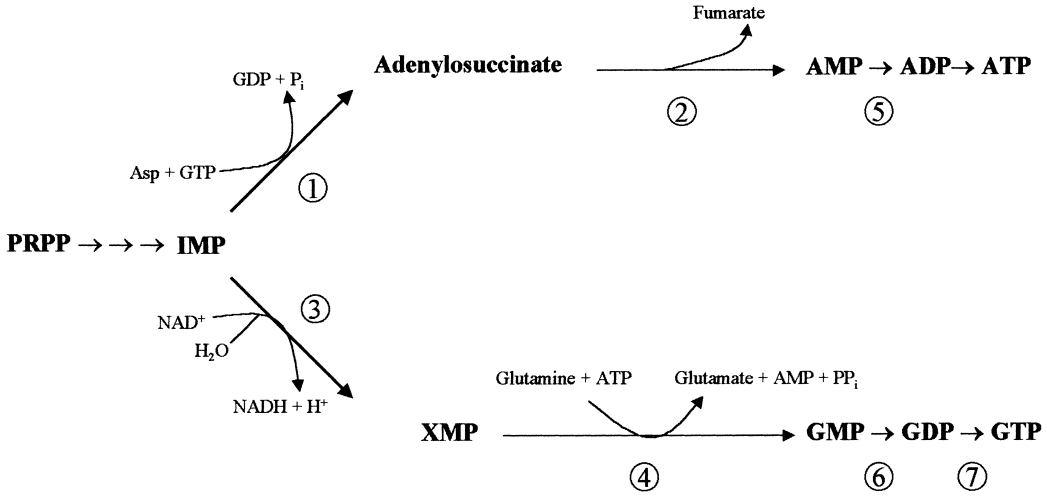
ATP may be formed from ADP, in several ways (PRPP synthetase and its reverse; oxidative phosphorylation; substrate level phosphorylation). GMP can be transformed into GDP and GTP by nucleoside monophosphate kinase (EC 2.7.4.8) and nucleoside diphosphate kinase (EC 2.7.4.6), respectively. These phenomena are represented in Scheme 1.

It is evident that nucleoside mono-, di-, and triphosphates are sequentially linked, and their formation is usually represented as a linear chain:

$$AMP \rightarrow ADP \rightarrow ATP$$

$$GMP \rightarrow GDP \rightarrow GTP.$$

Thus, we consider the synthesis of total adenylates (A = AMP+ADP+ATP) and total guanylates (G = GMP+GDP+GTP) cumulatively. This procedure is acceptable, if we assume that AMP and GMP mononucleotides are channeled as a constant



1 = Adenylosuccinate synthetase	EC 6.3.4.4
2 = Adenylosuccinate lyase	EC 4.3.2.2
3 = IMP dehydrogenase	EC 1.2.1.14
4 = GMP synthetase	EC 6.3.4.1
5 = Myokinase	EC 2.7.4.3
6 = Nucleoside monophosphate kinase	EC 2.7.4.8
7 = Nucleoside diphosphate kinase	EC 2.7.4.6

Scheme 1.

flux to ADP and GDP, ATP and GTP, which is a reasonable assumption in normal conditions, but must be verified in pathological situations.

In our previous experiments [1], we could not draw any conclusion about the 'inosinic branch point', and the subsequent transformations AMP→ADP→ATP and GMP→GDP→GTP in leukemia cells. In the present study, we followed the kinetic rate of incorporation of ^{14}C-formate not only into IMP, but also into AMP, GMP, ADP, GDP, ATP and GTP.

The aim of the present paper was to obtain more information on the nucleotide biosynthesis in lymphocytes of B-CLL patients, with a particular interest to the destiny of IMP at the inosinic branch point, and to the linear chains AMP→ATP and GMP→GTP.

2. Materials and methods

2.1. Chemicals

The nucleotides used as chromatographic standards were obtained from Sigma (St. Louis, MO, USA). The radiolabeled sodium-formate was obtained from Amersham (Amersham, UK); Lymphoprep was from Nycomed (Oslo, Norway). All other chemicals were of analytical grade from Merck (Darmstadt, Germany). All HPLC solvents were filtered through 0.45-μm membrane filters (Sartorius, Göttingen, Germany) prior to use.

2.2. lnstrumentation

For HPLC analysis, we used a Beckman chromatograph equipped with two model 110 B pumps, a model 166 UV detector set at 254 nm and a Whatman Partisil 10 SAX (4.6×250 mm) anion-exchange column. Identification of nucleotides and their quantification were carried out as previously described [1,4,5].

2.3. Patients

Five normal subjects (age 48–65 years) and five leukemia patients (60–75 years) were studied. The patients had a mean history of B-CLL of 2.5±0.5 years and attended the Hematological Division of Siena University Hospital for monthly check-ups. Diagnosis was performed on the basis of clinical and hematological findings and cytofluorimetric typing. At the time of blood sampling, the patients had not undergone chemotherapy in the previous 2 months. Informed consent was obtained from the healthy subjects and leukemia patients.

2.4. Preparation of total peripheral blood lymphocytes (PBL)

All blood samples were drawn in the morning (08.00 h) after overnight fasting; 60 ml of blood from normal subjects was usually sufficient for the determinations. In the leukemia patients, volumes proportional to the counts were used. The lymphocytes were isolated from whole blood by density floatation [6]. FACS analysis did not reveal platelet contamination. Lymphocytes, suspended in PBS to a final concentration of 10 000/μl, were counted in a Delcon cell counter. Cell viability was checked by the Trypan blue exclusion test: the number of dead cells was not significant.

2.5. Protein determination

Sonically treated lymphocyte extracts of each sample were prepared. Protein content of the extracts was evaluated according to Bradford [7], using bovine serum albumin as standard.

2.6. Kinetics of de novo synthesis of IMP and synthesis of other nucleotides

We evaluated the kinetics of IMP and of other nucleotides in the lymphocytes of healthy subjects and B-CLL patients, according to the indications of Gordon et al. [8]. After a pre-incubation period of 30 min, 2 mM glutamine and 0.5 mM ^{14}C-formate (25 μCi/mol) were added to the incubation buffer (pH 7.4) consisting of K_2HPO_4 (25 mmol/l), NaCl (100 mmol/l), HEPES (20 mmol/l), glucose (5.5 mmol/l), glycine (4 mmol/l), $NaHCO_3$ (10 mmol/l) and bovine serum albumin (0.4%).

It is known that the rate of purine synthesis in lymphocytes is dependent on a variety of factors, the most significant being the concentration of phosphate. Henderson et al. [9] demonstrated that purine

nucleotide synthesis in total leukocytes is stimulated by increasing the concentration of phosphate in the medium. The considerable increase probably reflects the availability of PRPP, the synthesis of which is activated by phosphate [10]. The use of 25 mM phosphate in our experiments, according to Gordon [8] has the advantage that the values of radioactivity incorporation is larger than would be the case if low phosphate concentrations were used: thus the sensitivity and reliability of the measurements are improved.

The lymphocytes were incubated for 17, 30, 60 and 154 min at a density of 6×10^6 cells/ml in an atmosphere of 5% CO_2 at 37°C. At the above times, 6×10^6 cells were taken and washed twice with 2 ml ice-cold PBS, then 100 μl ice-cold 0.4 M $HClO_4$ was added to the cell pellet. All the details of the preparation of perchloric extracts and the criteria of determination of nucleotides by HPLC have been reported in previous papers [1,4,5,9–12]. The peaks (corresponding to AMP, ADP, ATP, GMP, GDP, GTP and IMP) were collected directly in scintillation vials and counted for radioactivity in Instagel scintillant (added in 9/1 proportion to the collected fractions) in a Packard 1500 Tri-Carb liquid scintillation analyzer.

Since a gradient was used to resolve the nucleotides, we checked the quenching of the radioactivity at different buffer concentrations with standard solutions of ^{14}C-formate: no appreciable variations were observed.

The results were expressed in pmol of ^{14}C-formate incorporated into the nucleotides, per 10^6 cells and per mg of protein. The former is an absolute measure and the latter a relative one [1].

2.7. Evaluation of the inosinic branch point

The inosinic branch point was evaluated through the determination of radioactivity into IMP, AMP, GMP, and the calculation of IMP/AMP and IMP/GMP ratios. Low levels of these ratios are inversely related to the channeling of IMP into adenine or guanine mononucleotides, as shown in Scheme 1; therefore, they indicate that the conversion of IMP to AMP and GMP, respectively, is faster.

3. Results

In order to check the vitality of the cells during the incubation time, in a preliminary experiment, we evaluated the energy charge at the beginning and end of the incubation period, both for healthy and leukemia lymphocytes: it was 0.79 versus 0.77 in the former and 0.72 versus 0.70 in the latter. The energy charge was maintained, as previously reported [1].

Synthesis of IMP, AMP, ADP and ATP was lower in leukemia cells than in healthy lymphocytes. Fig. 1 shows IMP synthesis in lymphocytes of healthy sub-

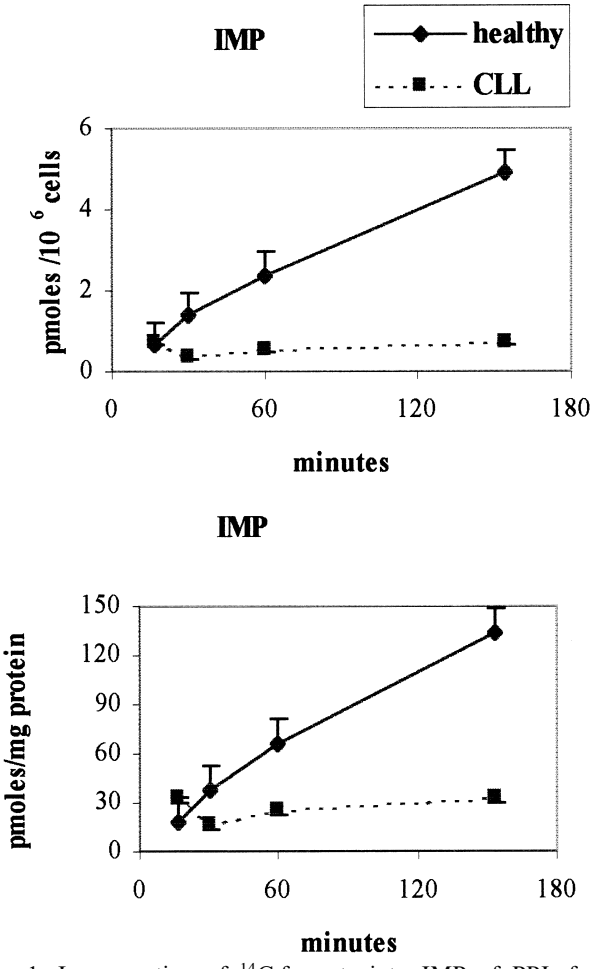


Fig. 1. Incorporation of ^{14}C-formate into IMP of PBL from healthy subjects and leukemia patients at different incubation times (see Section 2.6). The results are expressed in pmol of ^{14}C-formate incorporated into the nucleotides of 10^6 cells and into the nucleotides referred to 1 mg of protein. Bars indicate standard deviation.

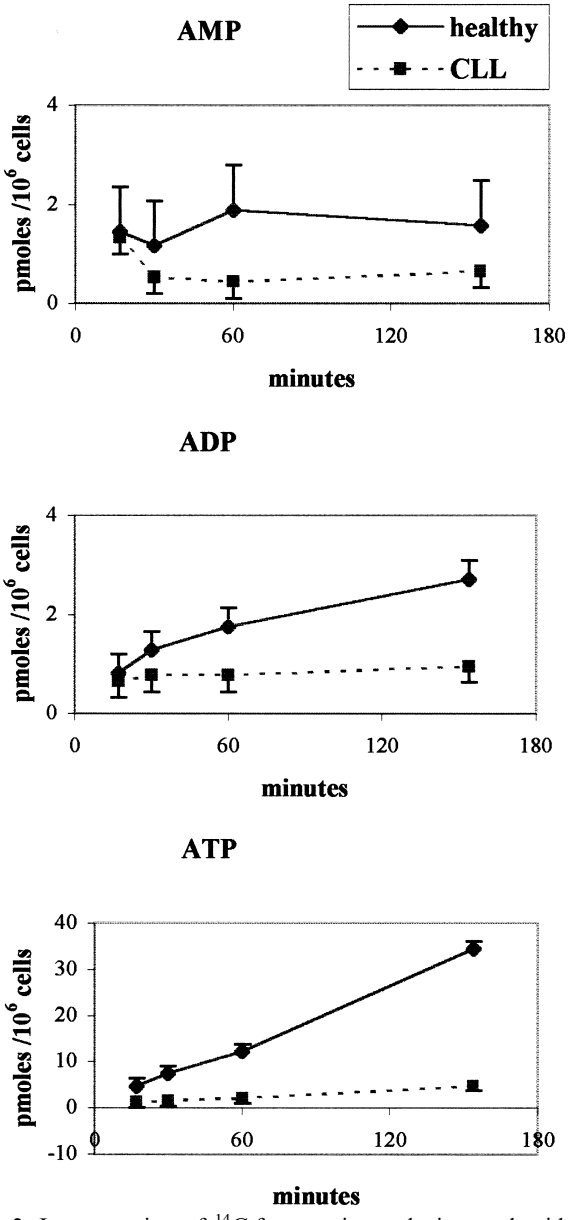


Fig. 2. Incorporation of ^{14}C-formate into adenine nucleotide of PBL from healthy subjects and leukemia patients at different incubation times (see Section 2.6). The results are expressed in pmol of ^{14}C-formate incorporated into the nucleotides of 10^6 cells. Bars indicate standard deviation.

jects and leukemia patients, expressed in pmol of ^{14}C-formate/10^6 cells and pmol/mg protein. Figs. 2 and 3 show synthesis of AMP, ADP, ATP.

The incorporation of ^{14}C-formate by GMP and GDP was not significantly different in lymphocytes from the two groups of subjects, but was lower for GTP, in the leukemia cells, when values were expressed in pmol/10^6 cells (Fig. 4). When results were expressed/mg protein, incorporation by GMP and GDP did not show significant variations in the leukemia cells, but was lower for GTP (Fig. 5).

The IMP/AMP and IMP/GMP ratios were calculated from the ^{14}C-formate incorporated into the nu-

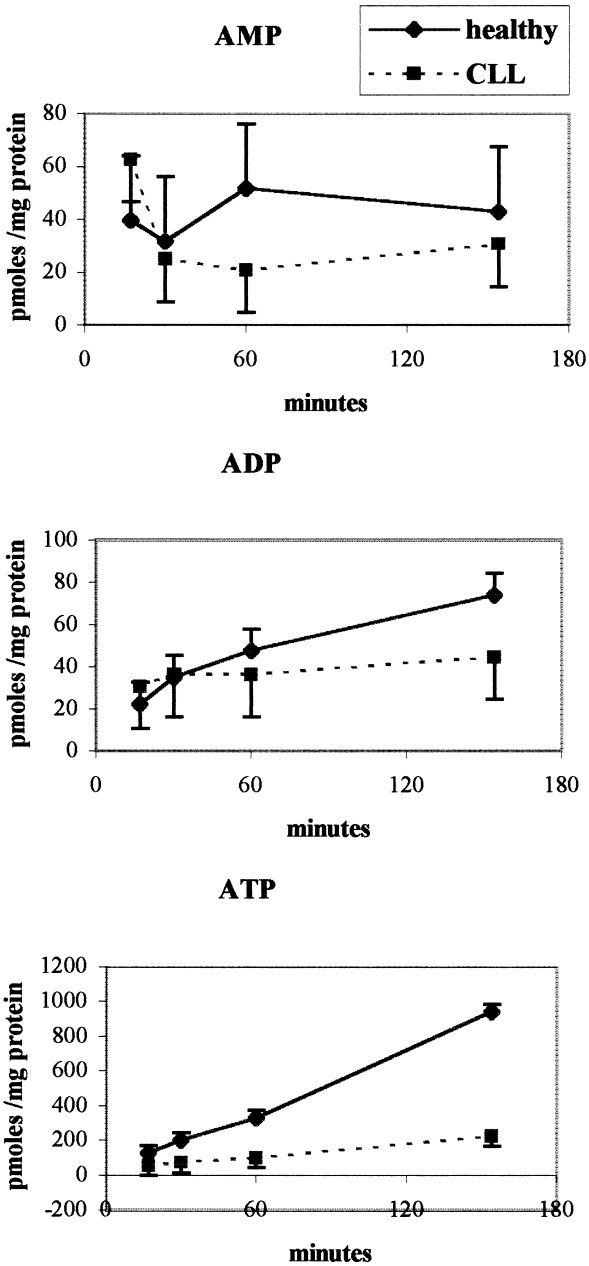


Fig. 3. Incorporation of ^{14}C-formate into adenine nucleotide of PBL from healthy subjects and leukemia patients at different incubation times (see Section 2.6). The results are expressed in pmol of ^{14}C-formate incorporated into the nucleotides and are referred to 1 mg of protein. Bars indicate standard deviation.

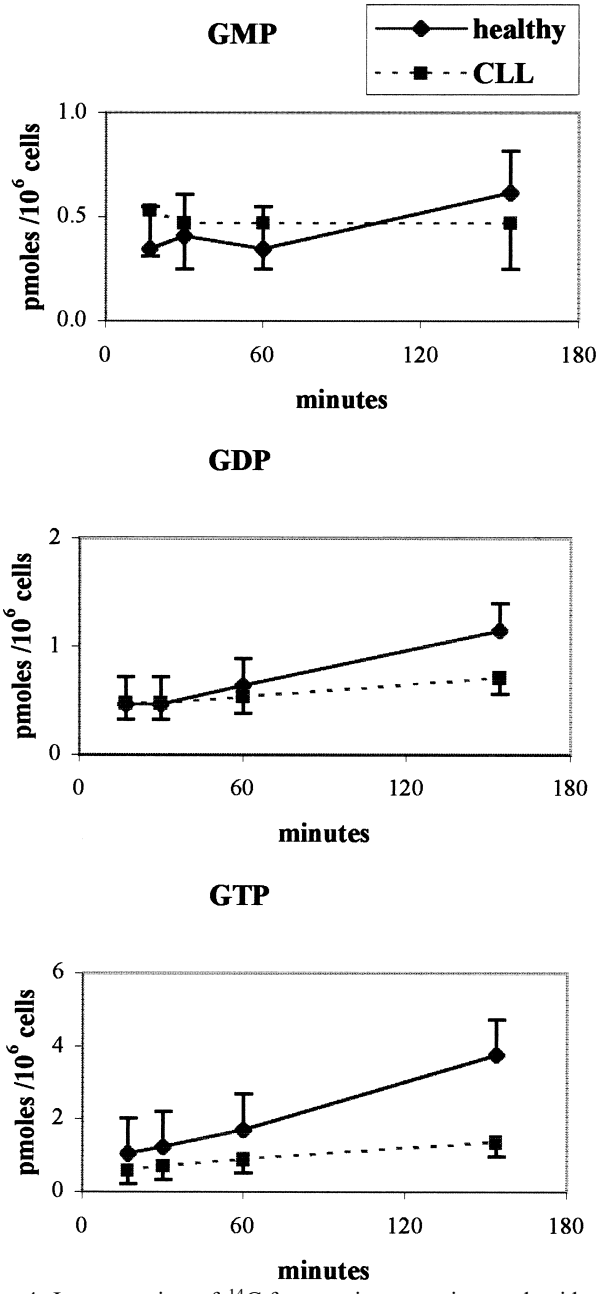


Fig. 4. Incorporation of ^{14}C-formate into guanine nucleotide of PBL from healthy subjects and leukemia patients at different incubation times (see Section 2.6). The results are expressed in pmol of ^{14}C-formate incorporated into the nucleotides of 10^6 cells. Bars indicate standard deviation.

cleotides of 10^6 cells (Table 1). The IMP/AMP ratio was lower in leukemia cells (1.09 versus 3.10 at 154 min); even higher was the decrease of the IMP/GMP ratio (1.51 versus 7.90 at 154 min).

The protein content shifted from an average of 36.40 ± 3.2 μg/10^6 cells to 21.00 ± 1.9 μg/10^6 cells in B-CLL lymphocytes (means ± S.E.).

4. Discussion

Some results of our present study deserve attentive scrutiny.

(1) The 'de novo' synthesis of IMP which occurs through the well-known PRPP → IMP sequence, with two points of ^{14}C-formate uptake, was found to be impaired in leukemia cells, both in absolute and relative terms. Ten reactions are involved in the transformation of PRPP into IMP. The reactions in which ^{14}C-formate is taken up by the precursors of IMP molecule are: (a) 5′-phosphoribosylglycinamide → 5′-phosphoribosyl-*N*-formylglycinamide; and (b) 5′-phosphoribosyl-4-carboxamide-5′-aminoimidazole → 5′-phosphoribosyl-4-carboxamide-5′-formamidoimidazole.

An interesting future task will be to ascertain which enzymes have lower activity in lymphocytic leukemia.

(2) At the 'inosinic branch point', AMP incorporated less ^{14}C-formate, however the results were expressed. In the case of GMP, no substantial difference was observed when the results were expressed per 10^6 cells; the variations expressed per mg protein were not significant.

A great deal of information can be deduced from the IMP/AMP and IMP/GMP ratios. These parameters, especially the latter, were depressed in leukemia cells. This means that even if the rate of IMP, AMP and GMP formation is lower in leukemia cells, as indicated by ^{14}C-formate incorporation, the channeling of IMP into AMP or GMP, at the 'inosinic

Table 1
Nucleotide ratios

	17 min	30 min	60 min	154 min
IMP/AMP				
Healthy subjects	0.44	1.20	1.26	3.10
Leukemia patients	0.53	0.66	1.20	1.09
IMP/GMP				
Healthy subjects	1.82	3.39	6.82	7.90
Leukemia patients	1.34	0.74	1.12	1.51

The ratios were calculated from the mean values of ^{14}C-formate incorporation into nucleotides.

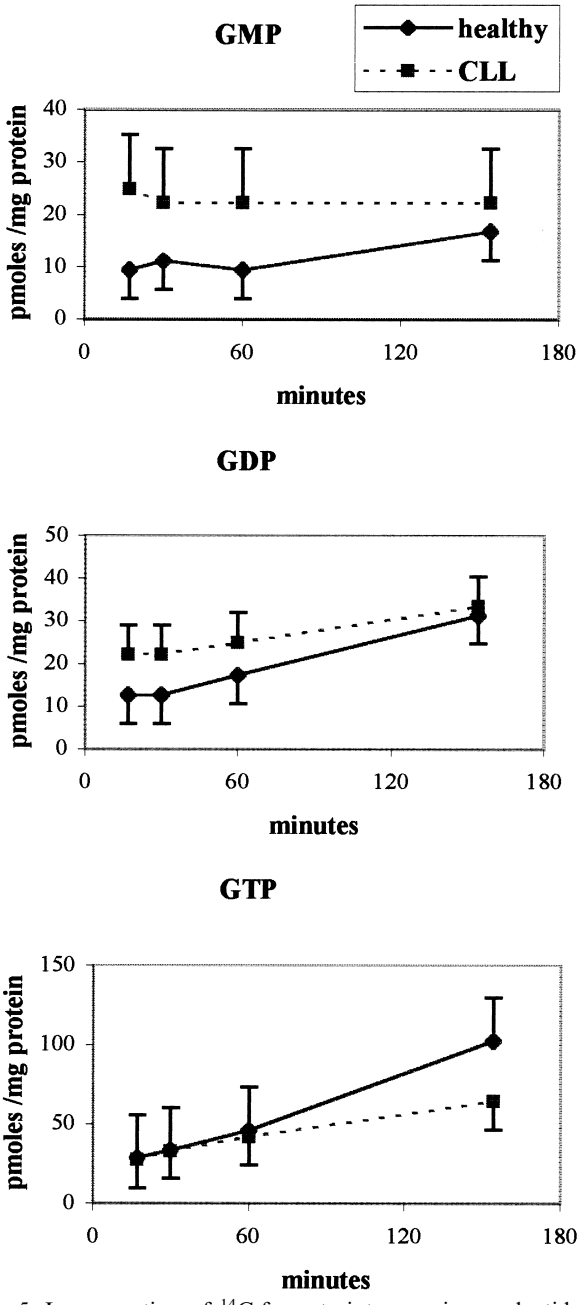


Fig. 5. Incorporation of ^{14}C-formate into guanine nucleotide of PBL from healthy subjects and leukemia patients at different incubation times (see Section 2.6). The results are expressed in pmol of ^{14}C-formate incorporated into the nucleotides and are referred to 1 mg of protein. Bars indicate standard deviation.

branch point', is relatively faster than in normal cells, especially for GMP. This phenomenon may compensate the lower synthesis of IMP, to ensure a satisfactory formation of nucleotides.

(3) On the basis of incorporation data, the entire sequence AMP→ADP→ATP is reduced in B-CLL lymphocytes, both when results are expressed per 10^6 cells and per mg protein.

The situation is partially different for guanylates. Only the last step of the sequence GMP→GDP→GTP is significantly lower in leukemia cells, as shown by the fact that GTP content (which is normally the most abundant of the guanine nucleotides) is the most depressed in leukemia cells [1], as well as its labeling after ^{14}C-formate, as shown by our present data.

(4) The present results are consistent with the previously reported analysis of nucleotide content in leukemia cells [1], in which all compounds were found to be lower. Di- and triphosphates were reduced in leukemia cells (ATP by 55% and GTP by 70%, when referred to 10^6 cells); the reduction was less evident if the data was expressed per mg protein.

(5) In the previous paper [1], we underlined the lower activity of all enzymes involved in nucleotide catabolism, the low and not significant increase in APRT and the 30% decrease in HGPRT, and we tried to understand the reduction in nucleotide content observed in leukemia cells.

Combining such results with the present data, we conclude that the decrease of the nucleotide content in leukemia cells seems to be only partially compensated, and we can better interpret such variations on enzymatic and kinetic basis.

(6) According to our precedent results [1], only IMP increased. The present research excludes that the IMP accumulation is linked to reduced channeling at the inosinic branch point, but is more probably due to the lower activity of IMPase [1].

The absolute levels of AMP, ADP and ATP, whenever they are referred to 10^6 cells or to proteins, are reduced [1]. The channeling of IMP towards AMP and total adenylates is relatively accelerated, as shown by the decrease of IMP/AMP ratio, but not enough to restore the normal values of adenine nucleotides.

The behavior of guanylates requires a specific comment. The levels of GMP, GDP and GTP, are lower in leukemia cells [1], with the GTP > GDP > GMP order. This is in agreement with the fact that we did not observe significant a difference between labeling of GMP and GDP, in the two

groups of subjects, and only a very low radioactivity in GTP in leukemia cells.

As shown by the values of IMP/GMP ratio, at the 'inosinic branch point', the channeling of IMP towards GMP and total guanylates is preferentially increased, even if only in relative terms. It is likely that this is not due to an enhanced number of enzyme molecules involved (IMP dehydrogenase and GMP synthetase), but to a reduced feed-back inhibition by the guanylates. However, the normal values are not restored, since the activity of HGPRT is impaired [1].

Bearing in mind all these data, the reduced nucleic acid synthesis [1] and the lower protein synthesis (which is GTP-dependent) demonstrated by the low protein content of leukemia cells, one may conclude that the reduced guanylate levels are due to a decreased synthesis, in absolute terms, and not to an increased utilization: the different behavior of GDP and GTP, can be better explained if we consider that they are formed by two different enzymes, the nucleoside monophosphate kinase (EC 2.7.4.8) and the nucleoside diphosphate kinase (EC 2.7.4.6), respectively.

Our research confirms the conclusion that it is always very important to understand the degree at which purine metabolism is impaired in leukemia cells and how this is reflected in nucleic acid synthesis. The enzymes involved in de novo synthesis, nucleotide catabolism and salvage pathway, their activity and structure, the corresponding genes, their regulation at metabolic and genomic level, deserve further consideration, for a better understanding of the life span of leukemia cells and of their viability: they may represent markers for precocious diagnosis, familiarity studies, for monitoring the disease; finally, they may represent the target for a rational chemotherapy.

The importance of guanylates must be specifically underlined. GTP controls the protein synthesis; the A/G ratio regulates the synthesis of nucleic acids; both phenomena are of extraordinary importance in the biology of the leukemia cells [4,13–15]. As shown by our experiments, guanylate metabolism seems to be specifically impaired in leukemia cells: there is a relatively increased channeling of IMP towards GMP; HGPRT and nucleoside di-phosphate kinase deserve further study, in order to understand the significance of their changes and the importance in the pathogenesis of leukemia.

The present research confirms the conclusion reached in our precedent paper [1], where we underlined the importance of studying concomitantly purine metabolism and its relationship with nucleic acid synthesis in leukemia cells.

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