Characteristic Abnormality of Deoxyribonucleoside Triphosphate Metabolism in Megaloblastic Anemia

By Nobuaki Iwata, Mitsuhiro Omine, Hiromasa Yamauchi, and Tadashi Maekawa

To elucidate the biochemical basis of megaloblastic hematopoiesis, the cellular content and metabolism of deoxyribonucleoside triphosphates (dNTPs) were investigated using the bone marrow cells from nine patients with untreated vitamin B12 deficiency and one with folic acid deficiency. The marked imbalance among four dNTPs was noted in all patients. dTTP was invariably elevated rather than depressed. The most striking abnormality, however, was the excessive accumulation of dCTP, which represented the consistent feature exclusive for megaloblastic anemia. Purine nucleotides were also involved to a lesser extent. The apparent turnover pattern of the dTTP pool of megaloblastic anemia marrow cells, in the presence or absence of hydroxyurea, did not differ significantly from that of normoblastic hematopoiesis. The megaloblastic cells assimilated exogenous thymidine into dTTP pool in vitro with enhanced efficiency. It was suggested that the excessive accumulation of dCTP may be related more closely to the pathogenesis of megaloblastic hematopoiesis than to the presumed but not proved deficiency of dTTP.

ONE OF THE MAJOR biochemical abnormalities in the megaloblastic hematopoiesis secondary to vitamin B12 deficiency or folate deficiency has been considered to be the impaired de novo thymidylate synthesis. Diminished suppression of 'H-thymidine (TdR) incorporation into DNA by the unlabeled deoxyuridine (UDr) \(^{1,2}\) and, transient hematologic improvement following TdR infusion \(^{4,6}\) have supported the concept that the megaloblastic bone marrow cells suffer from insufficient thymidylate supply for DNA synthesis. Hoffbrand and associates first examined directly the intracellular deoxyribonucleoside triphosphate (dTTP) contents of bone marrow cells from patients with this condition and showed that thymidine triphosphate (dTTP) content of untreated megaloblastic anemia did not differ significantly from acute myeloblastic leukemia. They also noted that the ratios of dTTP to deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dGTP), and deoxyytidine triphosphate (dCTP) were similar in these two conditions. \(^{3}\)

We have investigated in a similar way the dNTP metabolism of bone marrow cells from ten patients with megaloblastic anemia. The results confirmed the expanded rather than diminished dTTP pool in this condition. However, there were additional abnormalities in dNTP contents, among which the striking accumulation of dCTP was most consistent and characteristic for megaloblastic anemia. The turnover pattern of the dTTP pool did not indicate a retarded metabolism of dTTP. A remarkable expansion of the dTTP pool following exposure to TdR supported an active “salvage” pathway for thymidylate synthesis.

MATERIALS AND METHODS

Patients

Nine patients with untreated megaloblastic anemia secondary to vitamin B12 deficiency (PA) and one due to folic acid deficiency were subjected to the study. Vitamin B12 deficiency was either due to Addisonian pernicious anemia (7 cases) or total gastrectomy (2 cases). All patients were moderately or severely anemic (Hb: mean 6.1 g/dl, range 3.7–7.3 g/dl), and the bone marrow was frankly megaloblastic in all cases, with erythroid hyperplasia (E/G ratio: mean 1.2, range 0.7–1.7) at the time of investigation. Lowered serum concentration of either vitamin B12 or folate and impaired absorption of vitamin B12 were substantiated in all patients.

Four cases with hemolytic anemia associated with normoblastic hyperplasia (E/G ratio: mean 1.0, range 0.7–1.3) consisted of one patient with hereditary spherocytosis, one with congenital nonspherocytic hemolytic anemia, and two with acquired hemolytic anemia. Nine hematologically normal subjects served as control. The bone marrow cells from 18 patients with acute leukemias (15 myelogenous and 3 lymphatic) and 7 patients with chronic leukemias (5 myelogenous and 2 lymphatic) were also examined for comparison at the time of diagnosis or overt relapse.

Cell Preparation and dNTP Extraction

Approximately 5 ml of bone marrow aspirates were obtained into tubes containing ice-cold 0.25% EDTA in 0.85% saline solution. After being washed twice with cold 0.85% saline, the cells were subjected to hypotonic saline (0.23%) for 30 sec to remove red cells. Nucleated cells were finally suspended in Hanks’ balanced salt solution (BSS). Cell number was enumerated using a hemocytometer. Some 2 x 10^7 nucleated cells were centrifuged to tight pellet and the supernate was removed completely. Acid-soluble material was then extracted with 200 μl of ice-cold 0.5 M perchloric acid (PCA) for 20 min. After centrifugation, the resulting supernate was removed. PCA was neutralized by adding 20 μl of 4 M and 10–15 μl of 1M potassium hydroxide. The pH was adjusted to about 7.4 by adding 10 μl of 1 M potassium phosphate buffer, pH 7.4. The

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ABNORMALITY OF dNTP METABOLISM

The extract thus obtained was stored at \(-20^\circ\text{C}\) until assay. Our separate study has demonstrated the satisfactory recovery of dNTPs (approximately 95%) and the negligible effect of hypotonic treatment on the cellular dNTP content. No significant degradation of dNTPs has occurred over at least several months under the above storage condition. Smear preparations were made using final cell suspension by cytocentrifuge (Shandon-Elliot, England) and were stained with Wright-Giemsa stains in order to assess morphologically the proportion of immature (dividable) cells.

**Measurement of dNTPs**

The amount of dNTPs in the cell extract was determined by the enzymatic method of Solter and Handschumacher,\(^4\) with some modifications as described elsewhere.\(^5\) The method was satisfactory in terms of specificity for deoxy- and triphosphate compounds, sensitivity, and reproducibility.

The complete assay mixture contained 10 \(\mu\text{ mole}\) of Tris-HCl, pH 8.0, 0.5 \(\mu\text{ mole}\) of 2-mercaptoethanol, 0.5 \(\mu\text{ mole}\) of MgCl\(_2\), 1000 \(\mu\text{ mole}\) each of two unlabeled dNTPs (Sigma Chemical Co., St. Louis, Mo.), and 1.0 \(\mu\text{ Ci}\) (70-80 \(\mu\text{ mole}\)) of either \(^3\text{H-dCTP}\) or \(^3\text{H-dATP}\) (New England Nuclear, Boston, Mass.), 10 \(\mu\text{g}\) of heat-activated calf thymus DNA (highly polymerized, Sigma Chemical Co.) and 10 \(\mu\)l of cell extract in a final volume of 100 \(\mu\text{ l}\). Incubation was initiated by addition of 0.5 \(\text{U E. coli DNA polymerase (Kornberg I, Boehringer-Mannheim-Yamanouchi Co., Tokyo).}\)

After incubation at \(37^\circ\text{C}\) for 120 min in water bath, 20-\(\mu\)l aliquots of reaction mixture were applied in triplicate to Whatman GF/A filter discs (diameter 25 mm), which were presoaked with 10% trichloroacetic acid (TCA) immediately before application. Discs were then dried at \(40^\circ\text{C}\) for 20 min, and acid-soluble material was removed thoroughly by 3 changes of excess ice-cold 5% TCA containing 0.01 \(\text{M sodium pyrophosphate. Discs were subsequently rinsed in cold water and finally dipped in the chilled absolute ethanol.}\)

Each disc (diameter 25 mm), which were presoaked with 10% trichloroacetic acid (TCA) immediately before application. Discs were then dried at \(40^\circ\text{C}\) for 20 min, and acid-soluble material was removed thoroughly by 3 changes of excess ice-cold 5% TCA containing 0.01 \(\text{M sodium pyrophosphate. Discs were subsequently rinsed in cold water and finally dipped in the chilled absolute ethanol. Each disc was then transferred to the counting vial and dried prior to radioactivity measurement in the toluene scintillation system by a liquid scintillation spectrometer. The background radioactivity was obtained in each assay by preparing an assay mixture similarly treated except omission of cell extract. The parallel assay of 5 pmole dGTP was also performed to assure the reproducibility of the assay conditions. The radioactivity was converted to picomoles of dNTP by the chemically defined mixtures. The estimates were finally adjusted by calculation for the dilution of radioactive precursors by dNTPs present in the extract.\(^10\)

**Incorporation of TdR and UdR Into dTTP Pool**

In order to examine the ability of cells to assimilate exogenous nucleosides into dTTP pool, bone marrow cells suspended in Hanks' BSS containing 20\% dialyzed calf serum at a concentration of \(5 \times 10^6/\text{ml}\) were incubated in the presence of varying concentrations of TdR (0-0.2 mM) or UdR (0-3 mM) for 30 min. Vitamin B12 concentration of the undiluted dialyzed calf serum was 220 \(\mu\text{g/ml}\). At termination of incubation, cells were washed twice with cold Hanks' BSS and dNTPs were extracted as described above. For this experiment 5-10 \(\times 10^6\) cells per tube were used. When UdR was added as a precursor, effect of simultaneous addition of methotrexate (2 \(\mu\text{g/ml}\)) was also investigated.

**Turnover of dTTP**

Metabolic turnover pattern of intracellular dTTP pool was examined by determining the disappearance of prelabeled intracellular dTTP. Cells were incubated as described above at an approximate concentration of \(10 \times 10^6\) cells/ml with high specific activity \(^3\text{H-TdR (5 \(\mu\text{Ci/ml}, 0.2 \mu\text{M}) for 30 min to prelabel a portion of the dTTP pool. After being washed 3 times with excess cold Hanks' BSS, cells were resuspended in the prewarmed medium and further incubated at \(37^\circ\text{C}\) for up to 60 min prior to dTTP extraction. In some experiments, hydroxyurea was added at a final concentration of 4 \(\mu\text{M}\) at the start of the second incubation. The amount of \(^3\text{H-dTTP remaining in the extract was determined by measuring the radioactivity recovered in the acid-insoluble material in the similar assay system that contained 3 unlabeled dNTPs other than dTTP.}\)

**RESULTS**

**dNTP Pool Size of Bone Marrow Cells**

The dNTP pools of bone marrow cells from each group of patients are summarized in Table 1, and the data of individual patients are illustrated in Fig. 1. In normal bone marrow cells, dNTP pools were mostly within the range of several picomoles per \(10^6\) nucleated cells. Four dNTPs were not present at equimolar

<table>
<thead>
<tr>
<th>Specimen (%)</th>
<th>dTTP (pmole/1(10^6) Cells)</th>
<th>dCTP</th>
<th>dATP</th>
<th>dGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Megaloblastic anemia (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>18.8 ± 8.3</td>
<td>25.0 ± 11.9</td>
<td>27.8 ± 14.9</td>
<td>7.0 ± 3.9</td>
</tr>
<tr>
<td>Range</td>
<td>9.4-33.0</td>
<td>8.6-45.0</td>
<td>15.1-54.8</td>
<td>1.8-13.6</td>
</tr>
<tr>
<td><strong>Hematologically normal subjects (n = 9)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.4 ± 2.1</td>
<td>6.9 ± 3.4</td>
<td>1.9 ± 1.1</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>Range</td>
<td>4.0-11.0</td>
<td>3.4-13.6</td>
<td>0.5-3.2</td>
<td>0.1-3.3</td>
</tr>
<tr>
<td><strong>Normoblastic hyperplasia (n = 4)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>12.9 ± 2.4</td>
<td>7.5 ± 3.2</td>
<td>2.6 ± 0.7</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Range</td>
<td>10.0-16.7</td>
<td>3.7-12.6</td>
<td>1.4-3.1</td>
<td>0.9-4.0</td>
</tr>
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<td><strong>Acute leukemias (n = 18)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>—</td>
<td>17.1 ± 10.7</td>
<td>4.2 ± 2.7</td>
<td>4.1 ± 2.8</td>
</tr>
<tr>
<td>Range</td>
<td>—</td>
<td>2.1-38.6</td>
<td>0.8-9.4</td>
<td>0.4-9.6</td>
</tr>
<tr>
<td><strong>Chronic leukemias</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>—</td>
<td>4.8 ± 2.0</td>
<td>1.5 ± 0.6</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Range</td>
<td>—</td>
<td>2.1-8.2</td>
<td>0.7-2.6</td>
<td>0.4-2.1</td>
</tr>
</tbody>
</table>

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quantity, and in general, the dTTP content was largest in amount. When these data were compared between groups of disorders, it was evident that the dTTP content of megaloblastic anemia marrow cells was not reduced as compared with other conditions except acute leukemias, where the values varied considerably among patients. Moreover, this was accompanied by a consistent and striking increase of dCTP in megaloblastic anemia. A modest increase of dATP was also noted in this condition. However, the size of dGTP pool tended to be closer to that of normoblastic conditions. Such abnormalities in dNTP content of PA cells were essentially unaffected when compared with normal and normoblastic hyperplasia by adjusting the data according to the proportion of immature cells.

In the nonmegaloblastic condition, including leukemias, highly significant correlations were noted between dNTP pools; for instance, dTTP versus dCTP ($r = 0.88, p < 0.001$) or dATP versus dGTP ($r = 0.87, p < 0.001$). In contrast, the quantitative balance was perturbed markedly in favor of dCTP over dTTP in megaloblastic anemia. Likewise, in the latter condition, dATP was in a considerable excess to dGTP.

**Effect of Exogenous TdR and UdR**

The expansion of the dTTP pool following exposure of cells to added TdR at a concentration range of 2–200 μM was compared between PA, normoblastic hyperplasia, and acute myelogenous leukemia (Fig. 2). PA cells incorporated exogenous TdR into the dTTP pool with a far greater efficiency than the cells from normoblastic hyperplasia or acute myelogenous leukemia. In a similar experiment, the exposure of PA cells to UdR (0.25–3.0 mM) for 30 min resulted in a small increase of the dTTP pool in a concentration-dependent manner, but the simultaneous addition of 2 μM methotrexate prevented the expansion of dTTP pool (data not shown).

**Turnover Pattern of dTTP Pool**

The concentration of $^3$H-TdR used (0.2 μM) for prelabeling the dTTP pool did not alter the pool size to any appreciable extent. The disappearance curve apparently consisted of two exponential components, an initial rapid decline followed by a second slope with a more gradual decline (Fig. 3). The apparent T₁/2 of the initial component was approximately 7–10 min in two patients with PA examined. Addition of hydroxyurea resulted in a definite slowing of decline. Hydroxyurea inhibited the incorporation of $^3$H-TdR into DNA to 11% of control at 10 min and to 6.5%–7.8% thereafter up to 60 min under the experimental conditions employed. It was also noted that the treatment with
hydroxyurea did not abolish the disappearance of 3H-dTTP completely. The bone marrow cells from several other disorders, including acute and chronic myelogenous leukemias, sideroblastic anemia, erythro-leukemia, and hypersplenism, showed the basically analogous turnover pattern of the dTTP pool.

DISCUSSION

The widely appreciated 5-methyltetrahydrofolate trap hypothesis\textsuperscript{11,12} implies the impaired de novo thymidine nucleotide synthesis through the step of thymidylate synthetase due to inadequate supply of an active folate, 5,10-methylene tetrahydrofolate, the formation of which is indirectly dependent on vitamin B12 coenzyme.\textsuperscript{2,11,13} The beneficial effect of TdR infusion\textsuperscript{4,6} has supported the anticipation that the megaloblastic bone marrow cells would suffer from dTTP starvation, leading specifically to disordered DNA synthesis.

Hoffbrand et al. first demonstrated that the dTTP content of PA marrow cells was not reduced when compared with normal subjects and patients with acute leukemia.\textsuperscript{7} In this regard, our results are in agreement with these investigators. In fact, the dTTP pool of PA marrow cells was invariably expanded rather than diminished. Moreover, our observations revealed clearly the presence of additional disturbances in the dNTP content in this condition. The most striking and consistent abnormality was the excessive accumulation of dCTP. The abnormality was not limited to the pyrimidine nucleotides. dATP, in particular, was found to be affected, even though the deviation was less remarkable. These abnormalities were not ascribed solely to the increased proportion of immature cells. These findings were unique and characteristic of the untreated megaloblastic anemia among a spectrum of hematologic conditions examined thus far in our laboratory.\textsuperscript{9}

It seems likely that the elevated dCTP pool is due to shunting of pyrimidine deoxyribonucleotides toward dCTP owing to partially blocked thymidylate synthesis via deoxyuridylate. Under certain experimental conditions, the metabolic block of thymidylate synthetase step by antimetabolites has caused reduction of dTTP and dGTP along with elevation of dCTP and dATP in rat embryos, and human or animal cells in culture,\textsuperscript{14,15} although the results have often been variable in other cultured cells.\textsuperscript{16-18} However, the situation would be more complicated in human megaloblastic anemia, which represents the state of protracted metabolic disturbances where various compensatory mechanisms may be operative.

The method used in this study for dNTP quantitation does not discriminate strictly dTTP from deoxyuridine triphosphate (dUTP).\textsuperscript{14} However, UdR, added singly or in combination with methotrexate, failed to expand the "dTTP" pool to the extent comparable to that by TdR. This makes it unlikely that dUTP has affected estimation of the true dTTP pool at least to a significant degree. The dU TP pool of human lymphoid cells becomes expanded as a result of thymidylate synthetase block by methotrexate,\textsuperscript{19} whereas the dUTP pool was normally undetectable.\textsuperscript{19,20} The pool size of dUTP in the drug-inhibited cells was in the range of 0.2 pmole/10\textsuperscript{6} cells, even after 1000-fold expansion by the drug treatment.\textsuperscript{19} Likewise, it has been reported to constitute approximately 1% of dTTP in HeLa cells.\textsuperscript{21} It would be of interest to determine whether the dUTP pool is indeed increased in megaloblastic anemia to a magnitude so that it may bring about a significant cellular dysfunction. The expanded dUTP pool, if left unregulated, would lead to increased misincorporation of uracil into DNA in place of thymine in vivo.\textsuperscript{22} The
mammalian cells, including humans, normally possess an enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase), which is presumed to play a critical role in maintaining the dUTP level at the permissible level by specifically degrading dUTP to dUMP. It remains to be seen whether there occurs any abnormality in either dUTP pool or dUTPase in PA cells, as well as the uracil DNA-glycosylase, an enzyme responsible for the repair of misincorporated uracil. Any significant abnormality in any one of these would lead to the formation of structurally abnormal DNA, which may cause subsequent fragmentation of cellular DNA, a well known abnormality observed in megaloblastic anemia.

The activity of TdR kinase has repeatedly been shown to be elevated markedly in PA marrow cells and recently, also in the serum of PA patients. A highly efficient expansion of the dTTP pool in PA cells following exposure to relatively low concentrations of added TdR substantiates the activated salvage pathway of dTTP synthesis. Although the biochemical environment surrounding the bone marrow cells in situ is not precisely known, serum TdR concentration has been reported to be elevated in PA to the magnitude of 0.5 μM. Thus, a situation may exist in terms of increased availability of preformed TdR to PA marrow cells, particularly owing to enhanced intramedullary cell disintegration. The analogous situation may possibly apply to deoxycytidine, contributing to some extent to the increased dCTP.

The toxic effect of unbalanced dNTPs on cell proliferation and function has recently been impressively exemplified by the purinogenic immunodeficiency diseases. In these instances, purine nucleotides accumulate preferentially in the lymphoid system as a result of deficiency of one of the purine catabolizing enzymes, adenosine deaminase or purine nucleoside phosphorylase. Excessive dATP and dGTP are presumed to exert cytotoxicity through ribonucleotide reductase inhibition, when results in depletion of dCTP pool.

Despite the presence of striking abnormality in dNTP content, the precise mechanism of disturbed DNA synthesis, development of characteristic morphological changes, and subsequent premature cell death in megaloblastic anemia remains largely unclarified. Since our previous study has revealed that upon TdR infusion the reversion of megaloblastic to normoblastic hematopoiesis was preceded by a dramatic reduction of dCTP, together with simultaneous but transient further increment of dTTP, it appears plausible that at the florid stage of the disease, excessive dCTP rather than relative lack of dTTP is more intimately linked, at least phenomenologically, to the development of megaloblastic hematopoiesis.

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Characteristic abnormality of deoxyribonucleoside triphosphate metabolism in megaloblastic anemia

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