Thymidylate Synthetase Activity in Bone Marrow Cells in Pernicious Anemia

By Shinobu Sakamoto, Michiko Niina, and Fumimaro Takaku

The tritium release assay for the demonstration of thymidylate synthetase activity has been applied to the measurement of enzyme activity in the bone marrow of four patients with pernicious anemia and nine normal subjects. On the average, an approximately ninefold increase in enzyme activity was observed in patients with pernicious anemia. In the absence of 5,10-methylene-tetrahydrofolate, enzyme activity was reduced in both normal and in pernicious anemia cells. Addition of 5,10-methylene-tetrahydrofolate to the assay medium resulted in a far greater activation of thymidylate synthetase activity in megaloblastic bone marrow cells than in the cells of control subjects.

It is generally considered that the disturbance in DNA synthesis in vitamin B12-deficient mammalian cells is caused by impairment in the production of tetrahydrofolic acid, since vitamin B12 is required in the formation of tetrahydrofolic acid from 5-methyl-tetrahydrofolic acid. Inadequate amounts of tetrahydrofolic acid will result in insufficient production of thymidylate and, in turn, to a derangement of DNA synthesis.

In this study, we have measured the activity of thymidylate synthetase in bone marrow cells from patients with vitamin B12 deficiency megaloblastic anemia. This enzyme requires 5,10-methylene-tetrahydrofolate as coenzyme and is the key enzyme in de novo synthesis of thymidylate.

MATERIALS AND METHODS

Patients

Four untreated patients with megaloblastic anemia due to vitamin B12 deficiency were studied. As summarized in Table 1, all four patients showed remarkable megaloblastic erythropoiesis, and serum vitamin B12 levels were drastically reduced. Serum folic acid levels were within the normal range. Shilling test results were positive, and the patients responded well to vitamin B12 treatment. These data established the diagnosis of megaloblastic anemia due to vitamin B12 deficiency.

Preparation of Enzyme Solution

Bone marrow cells were obtained from the patients and nine healthy normal volunteers. Five to six milliliters of bone marrow blood cells were aspirated from the sternum or iliac crest into a sterile plastic tube containing a few drops of anticoagulant (Ancrod) and placed in the upright position at 4°C for 1-2 hr, after which the cell-rich plasma was collected and centrifuged. The cellular pellet was washed once with cold Tyrode’s solution and once with 0.05 M Tris HCl buffer, pH 8.0, containing 0.15 M KCl and 0.3 mM 2-mercaptoethanol. Any cell clots were removed by passage through a No. 150 mesh filter. The cells (adjusted to a concentration of 3-4 x 10⁷ cells per cu mm) were disrupted by rapid freezing and thawing five times and then centrifuged at
Table 1. Laboratory and Clinical Findings in Patients With Pernicious Anemia

<table>
<thead>
<tr>
<th>Case</th>
<th>Bone Marrow Myeloid/Erythroid Ratio</th>
<th>Serum Vitamin B12 (pg/ml)</th>
<th>Serum Folic Acid (L. Casei) (ng/ml)</th>
<th>Schilling Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Without I.F.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>With I.F.</td>
</tr>
<tr>
<td>1</td>
<td>0.51</td>
<td>36</td>
<td>9.3</td>
<td>0.13%</td>
</tr>
<tr>
<td>2</td>
<td>0.65</td>
<td>34</td>
<td>9.1</td>
<td>1.0%</td>
</tr>
<tr>
<td>3</td>
<td>0.97</td>
<td>35</td>
<td>9.2</td>
<td>1.63%</td>
</tr>
<tr>
<td>4</td>
<td>0.70</td>
<td>30</td>
<td>8.1</td>
<td>0.45%</td>
</tr>
</tbody>
</table>

Normal Range: Megaloblastic 350-1050\(^*\); 5.2-12.5\(^*\)
15%-30%

\(^*\)I.F., H.og intrinsic factor.
\(^1\)Results in 20 healthy subjects.
\(^2\)Results in 20 healthy subjects.

Serum vitamin B12 and folic acid levels were determined by radioassay\(^0\) and by microbioassay using Lactobacillus casei,\(^3\) respectively.

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105,000g for 60 min in a Hitachi 65P Ultracentrifuge.\(^4\) The clear supernatant was used for the assay of thymidylate synthetase activity.

**Assay of Thymidylate Synthetase Activity**

Thymidylate synthetase activity was measured by the tritium release assay using deoxyuridine monophosphate (dUMP)-5-\(^3\)H (Schwartz Bioresearch, 17.6 Ci/mM) as the substrate.\(^11-13\) The purity of this substrate was confirmed by paper chromatography. The incubation medium for assay of thymidylate synthetase contained, in 290 \(\mu\)l, dUMP-5-\(^3\)H, 50 nmoles (5 \(\mu\)Ci); 5,10-methylene-tetrahydrofolate, 720 nmoles, (supplied through the courtesy of Yamanouchi Pharmaceutical Co. Tokyo, Japan; the purity of this compound was confirmed by paper chromatography); ascorbic acid, 25 \(\mu\)moles; EDTA, 100 nmoles; 2-mercaptoethanol, 10 \(\mu\)moles; Tris-acetate buffer (pH 7.5), 50 \(\mu\)moles; and 100 \(\mu\)l of enzyme solution. Incubation was carried out in tubes wrapped with aluminum foil for 60 min at 37°C. The reaction was stopped with 50 \(\mu\)l of cold 40% TCA solution and the tube cooled in ice. Two milliliters of Norit A charcoal (150 mg/ml) were added, mixed vigorously, and the mixture centrifuged at 3000 \(g\) for 10 min. The supernatant was mixed with 20 ml of Bray’s solution\(^14\) and the radioactivity determined in a liquid scintillation spectrometer. Enzyme activity was expressed in pmoles of dUMP consumed per 5 \(\times\) 10\(^6\) cells per 60 min or tritium released (cpm) per 5 \(\times\) 10\(^6\) cells per 60 min.

**RESULTS**

**Assay of Thymidylate Synthetase in Human Bone Marrow Cells**

The enzyme reaction proceeded linearly with respect to enzyme concentration and time up to 60 min (data not shown), and the optimal pH for thymidylate synthetase from human bone marrow cells was distributed in a wide range from pH 6.8 to 8.5 (Fig. 1). The requirement for individual components in the assay medium was examined as shown in Table 2. Omission of 5,10-methylene-tetrahydrofolate reduced the enzyme activity in normal bone marrow cells to about 20% of that obtained in the complete assay system. Omission of other components did not influence enzyme activity. Thymidylate synthetase activity in bone marrow cells from the four cases of pernicious anemia was approximately nine times higher than that found in normal bone marrow cells (Table 3). In the absence of 5,10-methylene-tetrahydrofolate, the activity of thymidylate synthetase was reduced both in normal
and in pernicious anemia cells. In the presence of 5,10-methylene-tetrahydrofolate, however, the activity of thymidylate synthetase in the diseased bone marrow cells was far greater than that in normal bone marrow cells (Fig. 2). This result clearly showed that the thymidylate synthetase apoenzyme was present and even in excess in the bone marrow cells in pernicious anemia, but that its activity was not expressed due to a lack of folate coenzyme.

**Effect of Nucleoside Triphosphate on Thymidylate Synthetase Activity**

In order to study the effect of nucleoside triphosphate on thymidylate synthetase, 100 μmoles each of dTTP, dGTP, dATP, and dCTP were added to thymidylate synthetase from normal rat bone marrow cells. The addition of these four deoxynucleoside triphosphates had no inhibitory effect on the enzyme activity.

**Table 2. The Effect of Assay Conditions on Thymidylate Synthetase Activity in Normal Bone Marrow Cells**

<table>
<thead>
<tr>
<th>Omission</th>
<th>Thymidylate Synthetase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>107.7%</td>
</tr>
<tr>
<td>EDTA</td>
<td>80.3%</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>82.5%</td>
</tr>
<tr>
<td>5,10-methylene-tetrahydrofolate</td>
<td>20.5%</td>
</tr>
</tbody>
</table>

**Table 3. Thymidylate Synthetase Activity in Bone Marrow Cells**

<table>
<thead>
<tr>
<th>Case</th>
<th>Number of Cases</th>
<th>Range (pmoles dUMP Consumed per 5 x 10⁶ cells)</th>
<th>Mean ± SD (pmoles dUMP Consumed per 5 x 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>9</td>
<td>25.4–119.9</td>
<td>64.3 ± 31.4</td>
</tr>
<tr>
<td>Pernicious Anemia</td>
<td>4</td>
<td>370.5–690.6</td>
<td>552.1 ± 116.7</td>
</tr>
</tbody>
</table>
DISCUSSION

Several in vitro investigations of the incorporation of labeled precursors into DNA of bone marrow cells of megaloblastic anemia have shown that de novo synthesis of thymidylate was impaired, although salvage pathway synthesis, as indicated by the incorporation of \(^{3}\)H-thymidine into DNA, was active.\(^{19-21}\) Thymidine kinase, the rate-limiting enzyme in salvage pathway synthesis of thymidylate, has been demonstrated to have increased activity in the bone marrow cells of vitamin B\(_{12}\)-deficient patients.\(^{22,23}\)

Based on these findings, the 5-methyl-tetrahydrofolate trap hypothesis has been proposed.\(^{12}\) According to this hypothesis, formation of dTMP from dUMP in vitamin B\(_{12}\) deficiency is impaired because of the decreased thymidylate synthetase activity secondary to a relative deficiency of folate coenzyme.

Recently, Haurani reported decreased thymidylate synthetase activity in phytohemagglutinin-stimulated cultured lymphocytes from patients with pernicious anemia, while lymphocytes from patients with folic acid deficiency showed normal enzyme activity.\(^{24}\) The results of our present investigation, however, have shown that the amount of thymidylate synthetase apoenzyme in bone marrow cells from patients with pernicious anemia was not decreased, but markedly increased. The discrepancy in the results of the work of Haurani and our work could be due to the difference in the cells and/or to the method used for the measurement of enzyme activity. We used 5,10-methylene-tetrahydrofolate itself as coenzyme.

Activities of enzymes such as aspartate carbamyltransferase,\(^{25}\) dehydroorotase,\(^{25}\) ribonucleotide reductase,\(^{26}\) and thymidine kinase,\(^{22,23}\) all of which are involved in pyrimidine synthesis, have been reported to be elevated in peripheral blood or bone marrow cells from patients with pernicious anemia. According to the study of Smith et al.,\(^{25}\) the increased activities of aspartate carbamyltransferase and dehydroorotase and the normal activity of dihydroorotic dehydro-
genase found in cells from patients with pernicious anemia could not be explained by the immaturity of the cells in this disease, and a negative feedback control of enzyme concentration in blood cells was suggested.

In our studies, the activity of thymidylate synthetase was not suppressed by the addition of the deoxyribonucleoside triphosphates, and, therefore, a release of enzyme synthesis from end-product inhibition could not be considered as the cause of the observed increased activity. Moreover, the difference in the thymidylate synthetase activity between normal and anemic cells seemed too great to be accounted for by the immaturity of the bone marrow cells in pernicious anemia. It was, therefore, impossible to use this rationale to explain the observed response of the enzyme to the addition of 5,10-methylene-tetrahydrofolate.

We suggest, therefore, that the activity of thymidylate synthetase in bone marrow cells from patients with pernicious anemia is decreased in vivo because of a deficiency in folate coenzyme available to combine with protein apoenzyme, while the synthesis of the enzyme protein itself is stimulated to compensate for the decreased activity. This suggestion is supported by our previous demonstration of increased thymidylate synthetase activity in vitro in bone marrow cells from folic acid-deficient rats.\(^2\)\(^7\) As was the case in the present study, the increased thymidylate synthetase activity in these rats was dependent on 5,10-methylene-tetrahydrofolate. Therefore, it seems probable that in folate or vitamin \(B_12\) deficiency, thymidylate synthetase activity in bone marrow cells is decreased in vivo owing to the decrease in folate coenzyme, while the amount of enzyme protein itself is increased to compensate for this decrease. The mechanism of this compensation is unknown. It might be that the accumulation of dUMP within the folate coenzyme-deficient cells stimulates the synthesis of enzyme protein or inhibits its breakdown by a stabilizing effect. This speculation is supported by our observation of increased thymidylate synthetase activity in bone marrow cells incubated with deoxyuridine.\(^2\)\(^7\)

**ACKNOWLEDGMENT**

The present authors acknowledge the assistance of Drs. T. Maekawa and J. Tsuchiya in the department of Medicine, Gunma University, and to Dr. N. Tanaka in the department of Medicine, Jikei Medical School, in studying patients and measuring serum folic acid concentrations.

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