5-Methyltetrahydrofolate Related Enzymes and DNA Polymerase α Activities in Bone Marrow Cells from Patients with Vitamin B₁₂ Deficient Megaloblastic Anemia

By Yasuhiko Kano, Shinobu Sakamoto, Keita Hida, Keiichi Suda, and Fumimaro Takaku

The activities of 5-methyltetrahydrofolate (5-CH₃THF) related enzymes and DNA polymerase α were determined in bone marrow cells obtained from patients with vitamin B₁₂ deficient megaloblastic anemia and compared with those from healthy volunteers and patients with hemolytic anemia. 5-CH₃THF homocysteine methyltransferase activity was significantly lower than that in the control subjects. 5,10-methylenetetrahydrofolate reductase activity was only slightly elevated to that in the control subjects. DNA polymerase α activity was significantly higher than that in the control. High deoxuryridine suppression test values in vitamin B₁₂ deficient bone marrow cells were improved by tetrahydrofolate, but not by 5-CH₃THF. These data indicate that, even though the reverse reaction catalyzed by 5,10-methylenetetrahydrofolate reductase may be operative in vitamin B₁₂ deficiency, it is not sufficient to correct the disturbance in folate metabolism in vitamin B₁₂ deficiency. Increased DNA polymerase α activity may be due to compensation for disarranged DNA synthesis.

Although the precise mechanism for the impairment of folate metabolism induced by vitamin B₁₂ deficiency has not yet been established, most of the phenomena in impaired folate metabolism can be explained by the methylfolate trap hypothesis.7 The methylfolate trap hypothesis is based on the assumption that 5,10-methylenetetrahydrofolate reductase, which catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, does not catalyze the reverse reaction of this folate co-enzyme interconversion pathway.4 Recently, however, several reports have suggested that a vitamin B₁₂ independent pathway for the conversion of 5-methyltetrahydrofolate to tetrahydrofolate exists in the brain and platelets and that it plays a role in the methyl transfer from 5-methyltetrahydrofolate to aromatic amines.5,8 Thorndike et al.7 have reported that this pathway was also present in hematopoietic cells. The enzyme responsible for this pathway is considered to be 5,10-methylenetetrahydrofolate reductase. If this pathway actually operates in this manner, an escape hatch from the methylfolate trap can be established. However, 5,10-methylenetetrahydrofolate reductase activity has not been measured in vitamin B₁₂ deficient bone marrow cells.

Recently, Deacon et al.8 reported that in the deoxyuridine (dU) suppression test, tetrahydrofolate cannot correct the level of ³H-thymidine (³H-TdR) uptake in vitamin B₁₂ deficient bone marrow cells. This is also contradictory to the methylfolate trap hypothesis.

Vitamin B₁₂ deficiency leads ultimately to the impairment of DNA synthesis and cell proliferation.1,9 Although DNA synthesis is catalyzed by DNA polymerase α, β, and γ in mammalian cells, it is DNA polymerase α only that plays an important role in cell proliferation.10,11 This activity, however, has not been measured in vitamin B₁₂ deficient bone marrow cells.

Therefore, we have measured 5,10-methylenetetrahydrofolate reductase, 5-methyltetrahydrofolate homocysteine methyltransferase and DNA polymerase α activities in vitamin B₁₂ deficient bone marrow cells and have simultaneously performed the dU suppression test in order to investigate the effect of folate and vitamin B₁₂ derivatives on vitamin B₁₂ deficient bone marrow cells.

MATERIALS AND METHODS

Chemicals

5-¹⁴C-Methyltetrahydrofolate, ¹H-TdR and ¹H-dTTP were purchased from Radiochemical Center, Amersham, England. Other chemicals obtained were: S-adenosylmethionine, dithiothreitol, calf thymus DNA, dGTP, dCTP, dATP, dTTP, 5-methyltetrahydrofolate, tetrahydrofolate, and 2-deoxyuridine (dU) (Sigma, St. Louis, MO), cyanocobalamin, pteroylglutamate, EDTA, menadione, formaldehyde, dinedrome, and FAD (Wako, Tokyo, Japan), adenosylcobalamin and hydroxycobalamin (Nippon UCLAF, Tokyo, Japan); methylcobalamin (Eizai, Tokyo, Japan); 5-formyltetrahydrofolate (Japan Lederle, Tokyo, Japan), DL-homocysteine (Nakarai, Kyoto, Japan), and Hanks balanced salt solution (Nissui, Tokyo, Japan).

Patients

Eight patients with vitamin B₁₂ deficiency due to total gastrectomy, and three patients with vitamin B₁₂ deficiency due to pernicious anemia were studied (Table 1). All showed remarkable megaloblastic erythropoiesis, reduced serum vitamin B₁₂ levels, and normal serum folate levels. All responded well to vitamin B₁₂ treatment. The control subjects were healthy volunteers with normal serum vitamin B₁₂ and folate levels, and patients with hemolytic anemia with active erythropoiesis and normal serum vitamin B₁₂ and folate levels. Informed consent was obtained from normal subjects for bone marrow aspiration.

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Cell and Enzyme Preparation

Bone marrow blood was obtained using heparin as the anticoagulant. For the dU suppression test, nucleated cells were separated by Ficoll-Hypaque gradient centrifugation, washed three times with Hanks medium, and suspended at a density of 3 x 10^6 cells/ml in the same medium. For the enzyme assays, nucleated cells were separated by sedimentation at unit gravity through dextran sodium or 10 g 5-methyltetrahydrofolate plus 10 g methylcobalamin. dU was omitted in the control tube. Fifty g of sodium ascorbate were added to all tubes, and the final volume of each tube was adjusted to 2 ml with Hanks balanced salt solution. The 3H-TdR incorporation into DNA was counted in a Packard liquid scintillation spectrometer. Test values in the normal bone marrow cells were less than 10% (Table I).

The cell suspensions were subjected to four cycles of rapid freezing and thawing before being centrifuged at 100,000 x g for 60 min. The supernatants were used for the enzyme assays.

dU Suppression Test

The dU suppression test was carried out as described by Metz et al. with modification.

The assay culture tube contained 0.1 ml of bone marrow cells and 0.1 ml of each patient's serum plus 0.1 amole of dU with the addition of either 10 µg pteroylglutamate, 1 µg tetrahydrofolate, 10 µg 5-methyltetrahydrofolate, 1 µg 5-formyltetrahydrofolate, 10 µg methylcobalamin, 10 µg hydroxycoobalamin, 10 µg adenosylcobalamin, 10 µg cyanocobalamin, 1 mg methionine, 2 mg homocysteine and washed three times with phosphate buffered solution (PBS). For the assay of 5-methyltetrahydrofolate homocysteine methyltransferase, they were suspended at a density of 1 x 10^6 cells/ml in 50 mM potassium phosphate buffer, pH 7.2 with 1.5 mM reduced glutathione. For the assays of 5,10-methylenetetrahydrofolate reductase and DNA polymerase alpha, they were suspended at 1 x 10^6 cells/ml in sucrose TKM buffer (50 mM Tris HCl, pH 7.6, 25 mM KCl, 5 mM MgCl2, 5 mM dithiothreitol, 0.15 M sucrose, 2 mM phenyl-methylsulfonylfluoride and 0.1 mg/ml bovine serum albumin).

The cell suspensions were subjected to four cycles of rapid freezing and thawing before being centrifuged at 100,000 x g for 60 min. The supernatants were used for the enzyme assays.

Table 1. Summary of Patients With Vitamin B12 Deficient Megaloblastic Anemia.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (Y)</th>
<th>Hb (g/dl)</th>
<th>MCV (fl)</th>
<th>Serum Vitamin B12 (pg/ml)</th>
<th>Serum Folate (ng/ml)</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>60</td>
<td>5.3</td>
<td>113</td>
<td>65</td>
<td>5.3</td>
<td>pernicious anemia</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>62</td>
<td>5.8</td>
<td>124</td>
<td>79</td>
<td>8.4</td>
<td>pernicious anemia</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>42</td>
<td>5.0</td>
<td>114</td>
<td>126</td>
<td>7.4</td>
<td>pernicious anemia</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>46</td>
<td>8.5</td>
<td>133</td>
<td>138</td>
<td>7.5</td>
<td>postgastrectomy</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>73</td>
<td>8.7</td>
<td>106</td>
<td>84</td>
<td>10.0</td>
<td>postgastrectomy</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>52</td>
<td>9.7</td>
<td>138</td>
<td>100</td>
<td>7.2</td>
<td>postgastrectomy</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>70</td>
<td>5.5</td>
<td>99</td>
<td>100</td>
<td>6.2</td>
<td>postgastrectomy</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>56</td>
<td>9.5</td>
<td>102</td>
<td>112</td>
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<td>postgastrectomy</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>69</td>
<td>6.5</td>
<td>120</td>
<td>94</td>
<td>8.0</td>
<td>postgastrectomy</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>61</td>
<td>10.4</td>
<td>120</td>
<td>120</td>
<td>5.2</td>
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<td>M</td>
<td>71</td>
<td>9.3</td>
<td>135</td>
<td>84</td>
<td>17.4</td>
<td>postgastrectomy</td>
</tr>
</tbody>
</table>

* Serum vitamin B12: normal range 300–1000 pg/ml (L. Casei assay).
† Serum folate: normal range 3.4–14.4 ng/ml (L. Casei assay).

The assay culture tube contained 0.1 ml of bone marrow cells and 0.1 ml of each patient's serum plus 0.1 amole of dU with the addition of either 10 µg pteroylglutamate, 1 µg tetrahydrofolate, 10 µg 5-methyltetrahydrofolate, 1 µg 5-formyltetrahydrofolate, 10 µg methylcobalamin, 10 µg hydroxycoobalamin, 10 µg adenosylcobalamin, 10 µg cyanocobalamin, 1 mg methionine, 2 mg homocysteine or 10 µg 5-methyltetrahydrofolate plus 10 µg methylcobalamin. dU was omitted in the control tube. Fifty µg of sodium ascorbate were added to all tubes, and the final volume of each tube was adjusted to 1.0 ml with Hanks balanced salt solution. The tubes were then incubated at 37°C for 90 min. One µCi of 3H-TdR (specific activity 20 Ci/mmol) was added, whereupon the tubes were again incubated at 37°C for an additional 90 min. The cells were then washed with cold PBS, lysed to remove hemoglobin, and collected on Whatman GF/C filter discs. The discs were then washed with trichloroacetic acid. The 3H-TdR incorporation into DNA was counted in a Packard liquid scintillation spectrometer. Test values in the normal bone marrow cells were less than 10% (Table I).

Assay of 5-methyltetrahydrofolate homocysteine methyltransferase

The enzyme assay was carried out according to the method previously described except for the absence of vitamin B12 in the reaction mixture. The 5-methyltetrahydrofolate homocysteine methyltransferase activity was determined by measuring the incorporation rate of 3H-TdR into DNA of bone marrow cells and comparing it with that of control tubes without dU. The activity was calculated as the percentage of control activity and expressed as the percentage of control activity.

Table 2. dU Suppression Test Values in Vitamin B12 Deficient Megaloblastic Anemia (% control).*

<table>
<thead>
<tr>
<th>Case</th>
<th>2</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>Normal Range†</th>
</tr>
</thead>
<tbody>
<tr>
<td>dU</td>
<td>20.5</td>
<td>32.8</td>
<td>37.2</td>
<td>40.9</td>
<td>34.1</td>
<td>22.6</td>
<td>35.1</td>
<td>29.1</td>
<td>29.3</td>
</tr>
<tr>
<td>du + PteGlu</td>
<td>7.2</td>
<td>19.2</td>
<td>25.4</td>
<td>29.5</td>
<td>26.0</td>
<td>14.0</td>
<td>11.5</td>
<td>14.8</td>
<td>9.8</td>
</tr>
<tr>
<td>du + THF</td>
<td>—</td>
<td>10.8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>13.2</td>
<td>15.6</td>
<td>10.3</td>
<td>11.7</td>
</tr>
<tr>
<td>du + 5-CH3THF</td>
<td>18.3</td>
<td>19.5</td>
<td>38.0</td>
<td>40.1</td>
<td>31.2</td>
<td>19.6</td>
<td>29.7</td>
<td>21.2</td>
<td>21.3</td>
</tr>
<tr>
<td>du + 5-CH0THF</td>
<td>7.4</td>
<td>3.9</td>
<td>12.3</td>
<td>13.1</td>
<td>10.3</td>
<td>12.8</td>
<td>10.0</td>
<td>7.6</td>
<td>7.1</td>
</tr>
<tr>
<td>du + CH3B12</td>
<td>9.9</td>
<td>6.2</td>
<td>15.0</td>
<td>15.1</td>
<td>13.2</td>
<td>10.9</td>
<td>10.6</td>
<td>12.9</td>
<td>15.8</td>
</tr>
<tr>
<td>du + OHB12</td>
<td>11.6</td>
<td>5.2</td>
<td>15.6</td>
<td>16.3</td>
<td>12.1</td>
<td>11.0</td>
<td>13.3</td>
<td>12.6</td>
<td>15.4</td>
</tr>
<tr>
<td>du + AdB12</td>
<td>20.8</td>
<td>25.9</td>
<td>32.3</td>
<td>50.4</td>
<td>28.3</td>
<td>24.9</td>
<td>41.0</td>
<td>35.6</td>
<td>—</td>
</tr>
<tr>
<td>du + CNB12</td>
<td>9.8</td>
<td>12.0</td>
<td>26.3</td>
<td>34.4</td>
<td>22.6</td>
<td>9.1</td>
<td>14.6</td>
<td>16.4</td>
<td>—</td>
</tr>
<tr>
<td>du + 5-CH3THF + CH3B12</td>
<td>6.8</td>
<td>5.3</td>
<td>8.9</td>
<td>10.8</td>
<td>—</td>
<td>5.5</td>
<td>9.0</td>
<td>7.8</td>
<td>—</td>
</tr>
<tr>
<td>du + Met.</td>
<td>33.3</td>
<td>39.3</td>
<td>63.0</td>
<td>71.8</td>
<td>—</td>
<td>51.0</td>
<td>69.7</td>
<td>—</td>
<td>9.4</td>
</tr>
<tr>
<td>du + Homocysteine</td>
<td>21.2</td>
<td>27.9</td>
<td>32.3</td>
<td>35.3</td>
<td>—</td>
<td>—</td>
<td>27.8</td>
<td>—</td>
<td>5.4 ± 2.6</td>
</tr>
</tbody>
</table>

* dU suppression test was not performed in cases 1 and 7.
† mean ± S.D.
methyltransferase activity was expressed as nmoles of 14C-methionine formed per 1 x 10^8 immature cells per hr.

**Assay of 5,10-methylenetetrahydrofolate reductase**

The 5,10-methylenetetrahydrofolate reductase was determined according to the method described by Kutzbach et al.\(^4\) with a modification. The reaction mixture contained the following to a total volume of 250 µl: 0.2 M potassium phosphate buffer, pH 6.3; 60 mM according to the method described by Kutzbach et al.\(^4\) with a modification. The reaction mixture contained the following to a total volume of 250 µl: 0.2 M potassium phosphate buffer, pH 6.3; 60 mM FAD; 10 mM ascorbic acid; 1.6 mM EDTA; 480 mM 5-14C-methyltetrahydrofolate (1.8 x 10^3 dpm/nmole); 3.6 mM menadione (5 µl of a solution in 50% ethyl alcohol), and 100 µl of enzyme solution. The incubation was carried out at 37°C for 60 min whereupon the reaction was arrested by the addition of 0.25 ml of 0.6 M sodium acetate buffer pH 4.5, 0.1 ml of 0.1 M formaldehyde, and 0.15 ml of 0.4 M dimedone (dissolved in 50% ethyl alcohol). The mixture was heated at 100°C for 5 min, cooled at 0°C for 5 min, and then extracted with 2 ml of toluene. The radioactivity in a 1 ml aliquot of the toluene phase was determined by a liquid scintillation spectrometer. A mixture in which the enzyme solution was added after incubation was used as the control. The 5,10-methylenetetrahydrofolate reductase activity was expressed as nmoles of 14C-formaldehyde formed per 1 x 10^8 immature cells per hr.

**Assay of DNA polymerase α**

The DNA polymerase α activity was determined by using the reaction mixtures which contained the following to a total volume of 100 µl: 50 mM Tris HCl pH 7.2, 5 mM dithiothreitol, 50 mM KC1; 8 mM MgCl₂; 100 µg of bovine serum albumin, 2.5 µg of activated calf thymus DNA, 0.1 mM each of dGTP, dCTP, dATP and 3H-dTTP (1,800 dpm/nmole), and 50 µl of the enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by chilling in an ice bath. The aliquot was collected on Whatmann GF/C filter discs, which were then processed according to the method of Chang and Bollum.\(^5\) The radioactivity in the filter discs was counted in a toluene-based scintillator with a liquid scintillation spectrometer. Nonspecific endogenous activity was also assayed without activated DNA. The DNA polymerase α activity was expressed as nmoles of 3H-dTTP incorporated into DNA per 1 x 10^8 immature cells per hr. According to an analysis by sucrose density gradient fractionations of reaction products in this assay system, DNA polymerase activity is predominantly that of DNA polymerase α. Contamination of DNA polymerase β is less than 20%.

All enzyme assays were performed in duplicate.

**RESULTS**

**dU Suppression Test values**

As shown in Table 2, dU suppression test values were abnormal in all cases of vitamin B12 deficient megaloblastic anemia. These abnormalities were almost improved by the in vitro addition of tetrahydrofolate, 5-formyltetrahydrofolate, methylcobalamin and hydroxycobalamin. (Improvement is regarded as suppression of 3H-TdR incorporation into DNA to less than 15% of control value). The addition of pteroylglutamate and cyanocobalamin resulted in partial improvement, but 5-methyltetrahydrofolate, adenosylcobalamin and homocysteine had no effect on the improvement of abnormal dU suppression test values. The addition of both 5-methyltetrahydrofolate and methylcobalamin was more effective on the improvement of abnormal dU suppression test values on that of each agent. Methionine rather increased 3H-TdR incorporation into DNA.

The dU suppression test values of control subjects were all normal.

**Enzyme Activities**

The enzyme activities are expressed as the mean ± SD of nmoles of the product produced per hr. As shown in Fig. 1, the levels of 5-methyltetrahydrofolate homocysteine methyltransferase activity in bone marrow cells from patients with vitamin B12 deficiency (0.37 ± 0.42) were significantly lower than those in bone marrow cells from normal subjects (2.82 ± 0.89) and from patients with hemolytic anemia (3.26 ± 1.32). The differences were statistically significant (t = 6.28, df = 14, p < 0.001 and t = 5.12, df = 8, and p < 0.001, respectively). In contrast, as shown in Fig. 2, the levels of 5,10-methylenetetrahydrofolate reductase activity in vitamin B12 deficient bone marrow cells (25.59 ± 9.24) was higher than those in normal bone marrow cells (13.92 ± 3.96) (t = 4.21, df = 20, p < 0.001) and slightly higher than those in bone marrow cells from patients with hemolytic anemia (20.83 ± 6.16), but the differences
DISCUSSION

Our data on the dU suppression test values are different from those of Deacon et al. They reported that, in addition to 5-methyltetrahydrofolate, hydroxycobalamin and tetrahydrofolate were ineffective for the improvement of abnormal dU suppression test values in vitamin B12 deficient megaloblastic bone marrow cells; in the present study, however, hydroxycobalamin was effective for the improvement of abnormal dU suppression test values, as reported previously. Although tetrahydrofolate is slightly less effective for the improvement of abnormal dU suppression test values than 5-formyltetrahydrofolate, tetrahydrofolate is also effective for the improvement of abnormal dU suppression test.

The discrepancy in the results obtained between Deacon et al. and those we obtained may be due to the difference in methods employed in the dU suppression test. Deacon et al. used a modified method in which the control without dU was matched for each set of tests, and differed from the test only in that dU was omitted; that is, vitamin B12 and/or folate derivatives were also added to the control. Although the dU suppression test has been used in order to detect the deficiency in the 5,10-methylenetetrahydrofolate pool, with their methods, deficiency in the 5,10-methylenetetrahydrofolate pool of vitamin B12 deficient bone marrow cells is already corrected to some degree and the 3H-TdR uptake is decreased in these cells. Das et al. reported that the enhancing effect of folate and vitamin B12 derivatives on in vitro incorporation of 3H-dU into DNA by folate and/or vitamin B12 deficient cells, corresponded to their correcting effect on the abnormal dU suppression. Deacon et al. reported that the decrease in control values occurred in particular with the addition of tetrahydrofolate and 10-formyltetrahydr-
derivatives. These data suggest that these two folate derivatives may be effective for supplying 5,10-methylenetetrahydrofolate. Therefore, we think that the method employed by Deacon et al. is not suitable for investigating the effect of folate and vitamin B₁₂ derivatives on the incorporation of ³H-TdR into DNA by the vitamin B₁₂ deficient bone marrow cells.

5-methyltetrahydrofolate homocysteine methyltransferase activity was suppressed in vitamin B₁₂ deficient bone marrow cells, as previously reported. The levels of 5,10-methylenetetrahydrofolate reductase activity in vitamin B₁₂ deficient bone marrow cells were higher than those in normal bone marrow cells. However, there was no significant difference in 5,10-methylenetetrahydrofolate reductase activity between vitamin B₁₂ deficient bone marrow cells and bone marrow cells from patients with hemolytic anemia. While we expressed the enzyme activity per immature cell basis, it is possible that the difference between normal and vitamin B₁₂ deficient bone marrow cells is related to the heterogeneity of cell populations as well as the difference in the degree of cell maturation. In contrast, the levels of DNA polymerase α activity in vitamin B₁₂ deficient bone marrow cells were much higher than those in bone marrow cells from normal volunteers and patients with hemolytic anemia. These differences cannot be accounted for by the immaturity of vitamin B₁₂ deficient bone marrow cells. The activities of several enzymes such as thymidylate synthetase, thymidine kinase and 10-formyltetrahydrofolate synthetase have been reported to be elevated in vitamin B₁₂ deficient cells, to compensate for the depletion of folate coenzymes and pyrimidine. Conversely, serine hydroxymethylase activity was reported to be decreased in vitamin B₁₂ deficient bone marrow cells.

As shown in the present study, however, neither significant elevation nor decrease in 5,10-methylenetetrahydrofolate reductase was observed in vitamin B₁₂ deficient bone marrow cells, as was found to be the case in other cultured mammalian cells. This would suggest that 5,10-methylenetetrahydrofolate reductase does not play an important role in preventing the accumulation of 5-methyltetrahydrofolate in vitamin B₁₂ deficient bone marrow cells.

Thorndike et al. reported that in leukemic leukocytes, 5,10-methylenetetrahydrofolate reductase catalyzes the conversion of 5-methyltetrahydrofolate to 5,10-methylenetetrahydrofolate with the simultaneous nonenzymatic release of tetrahydrofolate and formaldehyde, which is, in turn, responsible for tryptofin formation. If this be the case in vitamin B₁₂ deficient bone marrow cells, it is difficult to explain the 5-methyltetrahydrofolate is not effective, whereas tetrahydrofolate is effective in the correction of the abnormal dU suppression test value. For that reason, the present study suggests that it is conceivable that this pathway of folate conversion is not sufficient to improve folate metabolism in vitamin B₁₂ deficient hematopoietic cells.

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