Defective DNA Synthesis in Human Megaloblastic Bone Marrow: Effects of Hydroxy-B_{12} 5'-Deoxyadenosyl-B_{12} and Methyl-B_{12}

By Martin B. Van Der Weyden, Mary Cooper, and Barry G. Firkin

In cobalamin deficiency, inadequate DNA-thymine synthesis appears to result from decreased conversion of N^5-methylenetetrahydrofolic acid to tetrahydrofolic acid (THF). The N^5-methyl THF conversion catalyzed by N^5-methyl THF-homocysteine methyltransferase requires a cobalamin coenzyme, presumed to be methylcobalamin (methyl-B_{12}). In support of the above, in B_{12}-deficient marrow cultures, methyl-B_{12} appears to be the most effective cobalamin form to correct defective DNA-thymine synthesis. This was measured by the ability of deoxyuridine to suppress tritiated thymidine incorporation into DNA. While methyl-B_{12} produced complete correction of defective DNA synthesis, 5'-deoxyadenosyl cobalamin (5'-deoxyadenosyl-B_{12}), cyanocobalamin (cyano-B_{12}), and hydroxocobalamin (hydroxy-B_{12}) effected only partial correction.

The methyl-B_{12}-mediated correction was blocked by methotrexate (MTX). The effect of MTX, in turn, was reversed by THF. In folate-deficient marrows, the B_{12} analogues did not correct defective DNA-thymine synthesis. The differential effects of hydroxy-B_{12} and methyl-B_{12} in correcting defective DNA-thymine synthesis in B_{12}-deficient marrows suggest that the complex mechanisms for N^5-methyl THF-homocysteine methyltransferase activation in Escherichia coli may not predominate in human hemopoietic tissue. Since methyl-B_{12} is the main component of plasma cobalamins, the critical determinant for megaloblastic maturation in B_{12} deficiency may be the delivery rate of methyl-B_{12} to marrow cells and its direct activation of N^5-methyl THF-homocysteine methyltransferase.

A RECURRENT ANOMALY of vitamin B_{12}- or folate-deficient megaloblastic marrow is interference in de novo DNA synthesis. This is

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Abbreviations used in text: cyano-B_{12}, cyanocobalamin; hydroxy-B_{12}, hydroxycobalamin; methyl-B_{12}, methylcobalamin; 5'-deoxyadenosyl-B_{12}, 5'-deoxyadenosyl cobalamin or coenzyme B_{12}; (Hydroxy-B_{12} is also called B_{12a} and has trivalent cobalt; B_{12e} is a one electron reduced form with divalent cobalt; B_{12a} is a two electron reduced form with monovalent cobalt.) dU, deoxyuridine; dUMP, deoxyuridine monophosphate; dTMP, thymidine monophosphate; THF, tetrahydrofolic acid; DHF, dihydrofolic acid; 9H-TdR, tritiated thymidine; MTX, methotrexate; PGA, folic acid.

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Fig. 1. Synthesis of DNA-thymine from deoxyuridine monophosphate (dUMP). Points of involvement of vitamin B12 and folate intermediates are indicated. dTMP, thymidine monophosphate; THF, tetrahydrofolic acid; DHF, dihydrofolic acid.

demonstrated by reduced incorporation of deoxyuridine (dU) into DNA-thymine.1-3 Added cyano-B12 results in partial correction of the reduced dU incorporation in B12- but not in folate-deficient marrows, whereas pteroylglutamic acid (PGA) completely corrects the defect in both types of deficient marrows.2,3 The interrelated biochemical pathways are shown in Fig. 1. N5-methyltetrahydrofolic acid (THF), which may accumulate in B12 deficiency,4 fails to correct the abnormal DNA synthesis in B12-deficient marrow5 unless B12 is added to the culture system.6 The conversion of N5-methyl THF to THF via homocysteine transmethylation involves a cobalamin-dependent methyltransferase7 and possibly a B12-independent reaction.6 In cobalamin deficiency, both cyano-B12 and hydroxy-B12 will satisfy in vivo B12 requirements in converting megaloblastic to normoblastic maturation.11 However, neither is a coenzyme form utilized intracellularly.12,18 Presumably, these B12 analogues must be metabolized into physiologically active forms, 5'-deoxyadenosyl-B12 or methyl-B12.

In man, methyl-B12, the predominant component of plasma cobalmins,13-17 is presumed to be the cobalamin coenzyme form in the N5-methyl THF-homocysteine methyltransferase reaction.12,18 5'-Deoxyadenosyl-B12 is the predominant component of hepatic cobalmins.19,20 acts as a coenzyme with methylmalonyl-CoA mutase in the conversion of L-methylmalonyl-CoA to succinyl-CoA.18,21 As a result, increased excretion of methylmalonic acid is a sensitive index of B12 deficiency.22 Since methyl-B12 is the major form of plasma cobalmins and is thought to be the cobalamin coenzyme in the cobalamin-dependent N5-methyl THF-
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Fig. 2. Effect of cyano-B₁₂, hydroxy-B₁₂, and PGA on deoxyuridine (dU) suppression of ³H-TdR incorporation into DNA in B₁₂-deficient marrows cultured exposed to daylight. Patients No. 1, open square; 2, open circle; 3a, open triangle; 3b, black triangle; 4, black circle; mean, unbroken line. dU, 10⁻¹ μmole; PGA, 50 μg; B₁₂ analogues, 1 μg; ³H-TdR, 1 μCi/culture.

homocysteine methyltransferase reaction, the current study was designed to determine, in B₁₂- or folate-deficient marrows, the differential effects of methyl-B₁₂, 5'-deoxyadenosyl-B₁₂, and hydroxy-B₁₂ on the conversion of dUMP to dTMP, an unequivocal folate-dependent step.²³⁻²⁷

MATERIALS AND METHODS

Effective synthesis of dTMP from dU in human marrow was measured by the ability of preincubation with unlabeled dU (at room temperature for 1 hr) to suppress incorporation into DNA of subsequently added ³H-TdR.¹² In megaloblastic marrow, defective DNA synthesis is demonstrable by reduced ability of dU to suppress incorporation of ³H-TdR into DNA.²³ For culture, 15–20 ml of marrow were aspirated from the posterior iliac

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Table 1. Summary of Patients Studied

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Serum Vitamin B₁₂ (pg/ml)</th>
<th>Serum Folate (ng/ml)</th>
<th>Bone Marrow*</th>
<th>dU Suppression of ³H-TdR Into DNA (%)</th>
</tr>
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<tbody>
<tr>
<td>B₁₂-deficient</td>
<td></td>
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<tr>
<td>1</td>
<td>Pernicious anemia</td>
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<td>12.4</td>
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<td>7.0</td>
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<td>48</td>
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<tr>
<td>3†</td>
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<td>4.7</td>
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<tr>
<td>4</td>
<td>Pernicious anemia</td>
<td>130</td>
<td>11.7</td>
<td>Mild</td>
<td>30</td>
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<tr>
<td>Folate-deficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Nutritional megaloblastic anemia</td>
<td>220</td>
<td>1.4</td>
<td>Moderate</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Alcoholic, chronic pancreatitis</td>
<td>340</td>
<td>2.4</td>
<td>Mild</td>
<td>26</td>
</tr>
</tbody>
</table>

*Degree of morphologic megaloblastic maturation.
†Studied on two separate occasions, 4 days apart.
crest directly into 10 ml of cold Hanks' solution containing heparin, 100 U/ml. All operations were carried out as previously described. The radioactive precursor used was thymidine (methyl-\(^3\)H, specific activity 15.1 Ci/mM; Radiochemical Centre, Amersham, England), prepared as a solution containing 10 μCi/ml. The dU load was 10\(^{-1}\) μmole/ml. The concentration of PGA was 50 μg/ml, THF (folinic acid), 150 μg/ml, the appropriate B\(_{12}\) analogues, 1 μg/ml; and the final concentration of MTX in the marrow cultures was 10\(^{-5}\) M. (The B\(_{12}\) compounds were generously supplied by Dr. L. Mervyn, Glaxo Laboratories, Greenford, England, through the courtesy of Dr. N. Gallagher, Department of Medicine, University of Sydney, Australia.) The cultures were performed in triplicate, and dU, PGA, and the appropriate B\(_{12}\) analogue were added to each set of cultures. When marrow incubations were performed in the dark, the culture tubes were wrapped in aluminum foil; addition of dU, B\(_{12}\) compounds, and \(^3\)H-TdR was also performed in the dark. \(^3\)H-TdR incubation (1 μCi/ml) was at 37°C for 2 hr, and DNA was extracted from the precipitate by the technique of Feinendegen et al., as modified by Cooper and Rubin. The radioactivity of the DNA extract was measured in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.), and results were expressed as total radioactivity incorporated into DNA. At the time of aspiration of bone marrow samples, venous blood was assayed for serum B\(_{12}\) and folate. The normal range of serum B\(_{12}\) is 170–700 pg/ml and that of serum folate is 3.0–15 ng/ml.

Six patients with megaloblastic bone marrow changes were studied. Four patients (No. 1–4) had B\(_{12}\) deficiency and two patients (No. 5 and 6) had folate deficiency. One patient (No. 3) was studied on two separate occasions. The serum vitamin levels, degree of megaloblastic marrow changes, and dU suppression effect are shown in Table 1.

**RESULTS**

*Effect of Cyano-B\(_{12}\), Hydroxy-B\(_{12}\) and PGA in B\(_{12}\)-deficient Marrow Cultures*

In normal marrow, added dU enters the dUMP→dTMP→DNA-thymine pathway so that the incorporation of \(^3\)H-TdR is reduced. The degree of diminution (suppression) of \(^3\)H-TdR uptake is a measure of dUMP incorporated into DNA. In normoblastic marrow cultures preincubated with dU at a concentration of 10\(^{-1}\) μmole/ml, subsequent \(^3\)H-TdR incorporation is diminished to 10% (or less) of control cultures, i.e., cultures not pre-
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Fig. 4. In vitro methotrexate (MTX) effect on the corrective effect of B12 analogues on the defective dU suppression of 3H-TdR incorporation into DNA in B12-deficient marrows cultured in the dark. Patients No. 1, black square; 2, open square; 3, dotted square. dU, 10⁻¹ μmole; B12 analogues, 1 μg; MTX, 10⁻⁵ M/culture.

incubated with dU.²⁻²⁸ This suppression implies normal DNA-thymine synthesis and is a sensitive index of tissue folate levels.³

The subnormal dU suppression of B12-deficient marrows cultured exposed to daylight was partially corrected toward the range obtained in normoblastic cultures by cyano-B12 (p <0.01) (Fig. 2). The change induced by added hydroxy-B12, at the same concentration, was more marked (p <0.01) than that produced by cyano-B12. Added PGA returned the dU suppressive effect to within the range obtained in normoblastic marrow cultures.

Effect of cyano-B12, Hydroxy-B12, Methyl-B12, and 5’-Deoxyadenosyl-B12 in B12-deficient Marrows Cultured in the Dark

The effect of cyano-B12 or hydroxy-B12 on the rate of conversion of dU to DNA-thymine in B12-deficient marrows was not significantly altered by culturing the marrows in the dark (p >0.1) (Fig. 3). 5’-Deoxyadenosyl-B12 resulted in a change of the abnormal dU suppression toward the normal range to the same degree as that produced by cyano-B12 (p >0.1), but its effect was less marked than that produced by hydroxy-B12 (p <0.01). Added methyl-B12 resulted in correction of the subnormal dU suppression to within the range obtained in normoblastic marrow cultures.

Effect of MTX on the Corrective Effect of Methyl-B12, 5’-Deoxyadenosyl-B12, Hydroxy-B12, and Cyano-B12 in B12-deficient Marrows Cultured in the Dark

The in vitro corrective effect of the B12 analogues on abnormal dU suppression of 3H-TdR incorporation in B12-deficient marrows did not occur in the presence of MTX (10⁻⁵ M) (Fig. 4). Added PGA in the presence of MTX was similarly without effect (Fig. 5), while incubation with THF produced normal dU suppression.

Effect of Methyl-B12 and 5’-Deoxyadenosyl-B12 in B12-deficient Marrows Cultured Exposed to Daylight

In this set of experiments, methyl-B12 and 5’-deoxyadenosyl-B12 were added to the marrow cultures in the dark. The cultures were then incubated exposed to daylight. The alterations in dU suppression induced by methyl-
Fig. 5. Effect of PGA and THF on in vitro MTX effect in B12-deficient marrows cultured in the dark. Patients No. 1, black square; 2, open square; 3, dotted square. dU, 10⁻¹ mole; PGA, 50 µg; THF, folinic acid, 150 µg; ³H-TdR, 1 µCi; MTX, 10⁻⁵ M/culture.

B12 or 5'-deoxyadenosyl-B12 in marrow cultures exposed to daylight, in contrast to their differential effect when cultured in the dark (Fig. 6A), were altered to the range produced by hydroxy-B12 (p < 0.05, Fig. 6B).

Effect of Cyano-B12, Hydroxy-B12, Methyl-B12, and 5'-Deoxyadenosyl-B12 in Folate-deficient Marrows Cultured in the Dark

The subnormal dU suppression of ³H-TdR incorporation into DNA-thymine in folate-deficient marrows is shown in Fig. 7. The subnormal dU suppression was not significantly altered by the addition of the four B12 analogues and was completely corrected by PGA.

DISCUSSION

In B12 deficiency, defective DNA synthesis has been attributed to a reversible deficiency of coenzymes essential in the pathways of deoxyribonucleotide synthesis—notably 5'-deoxyadenosyl-B12 or N⁵⁻¹⁰-methylene

Fig. 6. Effect of hydroxy-B12, methyl-B12, and 5'-deoxyadenosyl-B12 (coenzyme B12) on dU suppression of ³H-TdR incorporation into DNA in B12-deficient marrows cultured in the dark (6A) and in daylight (6B). dU, 10⁻¹ µmole; ³H-TdR, 1 µCi; B12 analogues, 1 µg/culture.
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Fig. 7. Effect of B<sub>12</sub> analogues on dU suppression of 3H-TdR incorporation into DNA in folate-deficient marrows cultured in the dark. dU, 10<sup>-1</sup> μmole; 3H-TdR, 1 μCi; PGA, 50 μg; B<sub>12</sub> analogues, 1 μg/culture.

THF, which is required for ribonucleotide reductase activity in Lactobacillus leichmannii, has been postulated to be an essential cofactor for ribonucleotide reduction in man. Recent evidence, however, suggests that this is not the case. Although increased ribonucleotide reductase activity occurs in human B<sub>12</sub>-deficient marrow preparations, no requirement for 5'-deoxyadenosyl-B<sub>12</sub> by human ribonucleotide reductase is demonstrable.

The alternative explanation for defective DNA synthesis in B<sub>12</sub> deficiency is the N<sup>5</sup>-methyl THF trap hypothesis in which decreased cellular levels of THF are ascribed to reduced N<sup>5</sup>-methyl THF-homocysteine methyltransferase activity. However, definitive studies on methyltransferase activities or N<sup>5</sup>-methylene THF or THF levels in human marrow deficient in B<sub>12</sub> are lacking.

dTMP for DNA-thymine synthesis can be formed from preformed TdR via the salvage pathway or from dUMP via the de novo pathway. In the de novo sequence, the donation of the one carbon unit to dUMP from N<sup>5</sup>-methylene THF results in oxidation of THF to DHF. Thus, in order for de novo dTMP synthesis to proceed, THF must be made available either by DHF reduction or by demethylation of N<sup>5</sup>-methyl THF. Consequently, THF must acquire the one carbon unit to become N<sup>5</sup>-methylene THF. Tissue THF availability may thus be limited by interference with the homocysteine-methionine pathway, which appears to depend on a cobalamin coenzyme.

Evidence for the N<sup>5</sup>-methyl THF trap hypothesis was demonstrated by Metz et al. N<sup>5</sup>-methyl THF did not correct abnormal conversion of dUMP to dTMP in B<sub>12</sub>-deficient marrows, while cyano-B<sub>12</sub> produced only a partial correction. PGA produced normal conversion.

The current study affords further evidence for the trap hypothesis. In the system utilized in this study, reduced de novo DNA-thymine synthesis in megaloblastic marrow appears to be due to interference with methylation of dUMP→dTMP, which is an unequivocally folate dependent step. Cyano-B<sub>12</sub> and 5'-deoxyadenosyl-B<sub>12</sub> produced comparable correction of defective dU→dTMP conversion in B<sub>12</sub>-deficient marrows. Hydroxy-B<sub>12</sub> induced a more marked correction of defective dU suppression of 3H-TdR incorporation, while methyl-B<sub>12</sub> reverted the abnormal dU suppression to levels obtained in normoblastic marrows. In folate-deficient marrow, the B<sub>12</sub> analogues were without effect. That the correction of defective dU suppression in B<sub>12</sub>-deficient
marrow by methyl-B₁₂ is mediated through changes in cellular levels of folate intermediates is suggested by the block of corrective effect of the B₁₂ analogues by MTX. The MTX effect could be reversed by simultaneous incubation of marrow cultures with THF.

The ineffective in vitro ability of cyano-B₁₂ and 5′-deoxyadenosyl-B₁₂ to correct defective dU–dTMP conversion contrasts with their in vivo effect.¹¹,³⁹ This may be a reflection of the relatively low conversion of cyano-B₁₂ to physiologically active forms,⁴⁰ of the difficulty of adenosylated compounds to traverse cell membranes, or of the degree of irreversibility of the B₁₂ to 5′-deoxyadenosyl-B₁₂ reaction.⁴¹,⁴²

The nature of the cobalamin coenzyme in the methyltransferase reaction in human hemopoietic cells is not established but is presumed to be methyl-B₁₂.¹²,¹⁸ In E. coli, the mechanism of the N⁵-methyl THF-homocysteine methyltransferase reaction involves binding of the cobalamin to methyltransferase and priming by S-adenosylmethionine to form methyl-B₁₂. After initial methylation, the cobalamin-enzyme complex can accept methyl groups from N⁵-methyl THF and can transfer them to homocysteine. A reducing system is required, and it has been suggested that the oxidation state of the cobalamin coenzyme involved is B₁₂s.⁸⁹

The effects induced by cyano-B₁₂, 5′-deoxyadenosyl-B₁₂, and particularly hydroxy-B₁₂ on defective dU to dTMP conversion in B₁₂-deficient marrow cultures suggest that the sequence of cobalamin-enzyme activation in E. coli may well occur in human hemopoietic tissue. However, it may not be the predominant mechanism, for it fails to take into account that the major component of plasma cobalamin is methyl-B₁₂, which in this study induced normal dU suppression in B₁₂-deficient marrow cultures. The methyl-B₁₂-methyltransferase complex in E. coli is photostable.⁴³ In this study, the culture of B₁₂-deficient marrows exposed to daylight with methyl-B₁₂ or 5′-deoxyadenosyl-B₁₂ produced correction of defective dU suppression in the range induced by hydroxy-B₁₂, the photolytic product of both methyl-B₁₂ and 5′-deoxyadenosyl-B₁₂.

The critical determinant in megaloblastic maturation in B₁₂ deficiency may be the delivery rate of methyl-B₁₂ to marrow cells and its direct activation of methyltransferase. This concept is supported by the efficacy of methyl-B₁₂ in correcting the abnormal dU suppressive effect in B₁₂-deficient marrows in this study and by the findings of Linnell et al.¹⁷ of reduced plasma methyl-B₁₂ levels, as compared with “hydroxycobalamin” components, in patients with pernicious anemia. The findings of the latter study, however, are at variance with those of Stahlberg, who demonstrated relative persistence of plasma methyl-B₁₂ and reduction in other plasma cobalamin components in pernicious anemia, with a similar disturbance in the ratio of methyl-B₁₂/5′-deoxyadenosyl-B₁₂ content in human B₁₂-deficient liver samples.¹⁵,²⁰

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