The Effect of Folate Analogues and Vitamin B\textsubscript{12} on Provision of Thymine Nucleotides for DNA Synthesis in Megaloblastic Anemia

By M. R. Taheri, R. G. Wickremasinghe, B. F. A. Jackson, and A. V. Hoffbrand

The role of vitamin B\textsubscript{12}, in the folate dependent biosynthesis of thymine nucleotides is controversial. In an attempt to clarify this, three methods have been used to assess the relative efficacy of vitamin B\textsubscript{12} (hydroxocobalamin) and various folate analogues in titrated concentrations at correcting ‘de novo’ thymidylate synthesis by megaloblastic human marrow cells: (1) The deoxyuridine (dU) suppression test which analyses the reduction in (\textsuperscript{3}H)-thymidine labeling of DNA by unlabeled dU. Marrow cells were also labeled with (6-\textsuperscript{3}H)-dU with assessment of (2) its incorporation into DNA and (3) the accumulation of (6-\textsuperscript{3}H)-deoxyuridine monophosphate (\textsuperscript{3}H-dUMP). The three methods gave similar results. In both, \textsuperscript{N}\textsubscript{5}-formyl tetrahydrofolate (formyl FH\textsubscript{4}) was the most effective agent at correcting thymidylate synthesis in megaloblastic anemia due to vitamin B\textsubscript{12} or folate deficiency. Vitamin B\textsubscript{12}, corrected the lesion in vitamin B\textsubscript{12} deficiency but not in folate deficiency. Tetrahydrofolate (FH\textsubscript{4}) and folic acid were effective in deficiency of vitamin B\textsubscript{12} or folate, although in both deficiencies they were less effective than formyl-FH\textsubscript{4}. Methyl–FH\textsubscript{4} was effective in folate deficiency but not in vitamin B\textsubscript{12} deficiency. These results confirm the failure of methyl–FH\textsubscript{4} utilisation in vitamin B\textsubscript{12} deficiency. They suggest that if vitamin B\textsubscript{12} is needed in the formylation of FH\textsubscript{4}, this is a minor role in provision of the correct coenzyme for thymidylate synthesis compared with its major role of provision of FH\textsubscript{4} from methyl–FH\textsubscript{4}.

At a biochemical level, megaloblastic anemia is probably due to defective DNA replication. Vitamin B\textsubscript{12} or folate deficiency is thought to result in reduced biosynthesis de novo of deoxythymidine monophosphate (dTTP) and so of deoxythymidine triphosphate (dTTP), one of the four immediate precursors of DNA.

In the ‘de novo’ synthesis of dTTP, the enzyme thymidylate synthase (5,10 methylene tetrahydrofolate: dUMP C–methyltransferase, E.C.2.1.1.45) catalyzes the conversion of dUMP to dTMP by the transfer of dUMP of a methyl group from the folate coenzyme, 5,10–methylene tetrahydrofolate (5,10–methylene FH\textsubscript{4}) probably in the polyglutamate form. This reaction occurs at a reduced rate in folate or vitamin B\textsubscript{12} deficiency. This biochemical lesion has previously been demonstrated indirectly by the deoxouridine (dU) suppression test. In this, DNA of bone marrow cells is labeled with (\textsuperscript{3}H)–thymidine (\textsuperscript{3}H–TdR) via the ‘salvage’ pathway. In a second incubation, nonradioactive dU at high concentration is added. This inhibits (\textsuperscript{3}H)–TdR uptake due, in part at least, to competition by thymine nucleotides synthesized ‘de novo’ from the ‘cold’ dU. The dU suppression is expressed as the (\textsuperscript{3}H)–TdR incorporation into DNA in the presence of dU as a percentage of the (\textsuperscript{3}H)–TdR incorporation in the absence of dU. Megaloblastic marrow cells give higher values than normal due to the impaired conversion of dU to thymine nucleotides although other factors may be important. Addition of vitamin B\textsubscript{12} or folate analogues to megaloblastic marrows restores dU suppression to normal value in a manner specific for the appropriate vitamin deficiency, making the test of considerable diagnostic importance.

In a previous study, we have shown that defective synthesis of thymine nucleotides can be demonstrated in megaloblastic anemia more directly by measuring the incorporation of (\textsuperscript{3}H)–dU into DNA of bone marrow cells ((\textsuperscript{3}H)–dU incorporation test). There is a massive accumulation of (\textsuperscript{3}H)–dUMP in megaloblastic cells and DNA labelling is increased and (\textsuperscript{3}H)–dUMP accumulation reduced by the addition of vitamin B\textsubscript{12} and \textsuperscript{N}\textsubscript{5}–formyl tetrahydrofolate (formyl FH\textsubscript{4}) to megaloblastic cells.

In the present study we show that the pattern of correction of megaloblastic marrow cells by vitamin B\textsubscript{12} and folate compounds is reflected with equal specificity in the dU suppression test and by the (\textsuperscript{3}H)–dU incorporation method. The combined tests have been used to analyse the effect of vitamin B\textsubscript{12} deficiency on folate metabolism, in particular whether tetrahydrofolate (FH\textsubscript{4}) itself, which bypasses the ‘methyl tetrahydrofolate trap’ in vitamin B\textsubscript{12} deficiency, is also effective in correcting thymidylate synthesis or whether formyl–FH\textsubscript{4} is needed. A brief abstract of some of this work has been published.

MATERIALS AND METHODS

Reagents

(5–Me–\textsuperscript{3}H)–thymidine (5 Ci/mmole) and deoxy (6–\textsuperscript{3}H)–uridine (20 Ci/mmole) were from The Radiochemical Centre, Amersham, Bucks, UK. Both preparations had a radiochemical purity of 99% as determined by four chromatographic systems. The absence of signif-
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was inferred by the observation that incorporation of (6-^3H)-dU into DNA of lymphocytes was totally abolished by methotrexate. Deoxyuridine was from Sigma Chemical Company, Fancy Road, Poole, Dorset, BH17 7NH. Tetrahydrofolic acid (Sigma, 90% pure) was dissolved in 1% potassium ascorbate, pH 6.5 and stored under nitrogen. Comparison of the biological activity of old and new batches of this compound showed that it was stable for at least 8 mo under these conditions. dl-5-methyltetrahydrofolic acid (barium salt, Sigma, 95% pure), was dissolved in isotonic saline immediately before use. dl-N^3-formyltetrahydrofolic acid (calcium salt) was from Lederle Laboratories, and Vitamin B12 (hydroxocobalamin, Neo-cytamen) was from Glaxo, Greenford, Middlesex, UK. The latter two preparations are stable in the dark at room temperature.

The sources of other reagents have been listed previously.

Marrow Samples

Bone marrow samples were obtained by aspiration from the posterior iliac crest of 25 patients with megaloblastic anemia and from 14 normal volunteers with their informed consent. Patients were classified as being vitamin B12 deficient, folate deficient, or both (with low serum levels of both vitamins) on the basis of routine microbiological assay of their serum vitamin B12 and folate. Normal ranges established in this laboratory are: serum vitamin B12, 160-925 ng/l (E. gracilis assay) and serum folate, 3-20 mu/l (L. casei microbiological assay).

Marrow cells were freed from erythrocytes as described and resuspended in Hanks’ salt solution supplemented with 20% autologous serum for use.

**Results**

Bone marrow samples were obtained by aspiration from the posterior iliac crest of 25 patients with megaloblastic anemia and from 14 normal volunteers with their informed consent. Patients were classified as being vitamin B12 deficient, folate deficient, or both (with low serum levels of both vitamins) on the basis of routine microbiological assay of their serum vitamin B12 and folate. Normal ranges established in this laboratory are: serum vitamin B12, 160-925 ng/l (E. gracilis assay) and serum folate, 3-20 mu/l (L. casei microbiological assay).

**Normal Bone Marrow**

Addition of 1 mM dU to 14 normal bone marrows suppressed incorporation of (H)–TdR into DNA to a mean value of 3.4 (±2.1 S.D) % of the (H)–TdR incorporation in the absence of dU (Fig. 1). A value of 8% or less in the dU suppression test is therefore taken as normal. Incorporation of (H)–dU into DNA of normal bone marrows ranged from 48,105 to 374,833 (mean 178,096) dpm.10^6 cells.h^-1 incubation. The incorporation was not appreciably altered by the addition of vitamin B12 or of folate analogues (Fig. 2). The absolute accumulation of (H)–dUMP in untreated normal marrow cells was extremely small compared to that in untreated megaloblastic marrow and ranged from 2,017 to 8,712 (mean 4,151) dpm.10^6 cells. The accumulation was reduced to 18 (±12)% of control values by addition of formyl–FH4 (Fig. 3).

**Megaloblastic Bone Marrow**

In megaloblastic bone marrow, the mean dU suppression value was 17 (±5.1)%. Incorporation of (H)–dU into DNA was similar to normal and ranged from 60,283 to 388,808 (mean 203,357) dpm.10^6 cells but accumulation of (H)–dUMP was greatly raised compared to normal ranging from 49,106 to 709,883 (mean 515,004) dpm.10^6 cells.
Addition of Vitamin B12 or Folate Analogues to Vitamin B12- or Folate-deficient Bone Marrow. Vitamin B12 (Hydroxocobalamin)

In the dU suppression test, addition of 100 μg.ml⁻¹ of vitamin B12 to 12 vitamin B12-deficient marrows reduced the abnormal suppression of (³H)-TdR incorporation from a mean of 17 (±6.5)% to a mean of 8 (±4.3)% (Fig. 1). Vitamin B12 was effective at levels as low as 10 μg.ml⁻¹ (Table 1). Incorporation of (³H)-dU into DNA also showed an increase to a mean of 244 (±99)% on addition of 10 μg.ml⁻¹ of vitamin B12 compared to the incorporation by untreated cells (Fig. 2). In the same experiment the accumulation of (³H)-dUMP was decreased (mean 60 (±34)% of untreated cells) by addition of vitamin B12 (Fig. 3). Thus addition of vitamin B12 improved all three tests towards normal in vitamin B12-deficient marrow.

In contrast, addition of vitamin B12 to folate-deficient marrows did not correct the dU suppression test (Fig. 1), increase (³H)-dU incorporation (Fig. 2) or decrease (³H)-dUMP accumulation (Fig. 3).

N⁵-formyl Tetrahydrofolate (Formyl-FH₄)

Among the folate analogues tested, formyl-FH₄ was the most effective in correcting the abnormal test results in both vitamin B12 and folate deficient megaloblastic bone marrows. Formyl-FH₄ at concentrations from 0.3 μg.ml⁻¹ to 30 μg.ml⁻¹ caused a marked correction in dU suppression in vitamin B12-deficient marrows from a mean of 17 (±6.4)% to a mean of 1.8 (±0.8)% (Fig. 1, Table 1). In the (³H)-dU incorporation test, formyl-FH₄ (30 μg.ml⁻¹) increased DNA labeling in DNA to 350 (±196)% of that of untreated cells (Fig. 2) and reduced (³H)-dUMP accumulation sharply to a mean of only 4.9 (±2.9)% of the control value (Fig. 3). Both these effects in the (³H)-dU incorporation test were evident at formyl-FH₄ concentrations as low as 6 μg.ml⁻¹ (Fig. 4).

Addition of 30 μg.ml⁻¹ formyl-FH₄ to folate deficient marrows also resulted in a marked correction of dU suppression from 15.8% to 1% (Fig. 1). As little as 0.3 μg.ml⁻¹ of formyl-FH₄ gave correction to values within the normal range (Table 1). (³H)-dU incorporation was also increased (Fig. 2) and (³H)-dUMP accumulation was reduced (Fig. 3).

Tetrahydrofolate (FH₄)

Addition of 30 μg.ml⁻¹ of FH₄ corrected the dU suppression of vitamin B₁₂-deficient marrows from 17 (±6.4)% to 5.4 (±2.4)% (Fig. 1). FH₄ was less effec-
Table 1. Effect of Varying Concentration of Vitamin B₁₂ or Folate Coenzymes on the dU Suppression Test in Megaloblastic Bone Marrow Cells.

<table>
<thead>
<tr>
<th>Vitamin B₁₂ Deficiency</th>
<th>Folate Deficiency</th>
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<tbody>
<tr>
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<td>No.</td>
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<tr>
<td>(³H)-TdR uptake</td>
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<td>+ dU (1 μm) alone</td>
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<tr>
<td>% of Control</td>
<td>10</td>
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<tr>
<td>+ dU + vit B₁₂ 100μg/ml</td>
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<td>+ dU + formyl FH₄</td>
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<td>10μg/ml</td>
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<td>+ dU + folic acid</td>
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Effect of varying concentrations of vitamin B₁₂ or folate coenzymes on the dU suppression test in megaloblastic bone marrow cells.

At 30 μg.ml⁻¹, FH4 was nearly as efficient as formyl-FH₄ in reducing the dU suppression test value in folate deficiency (mean 2.4 (±0.4)%). The same correction was observed at 3 μg.ml⁻¹ FH₄ but higher concentrations gave suppression values above the normal range and above the value found with equivalent concentrations of formyl-FH₄ (Table 1). Incorporation of (³H)-dU into DNA was increased by addition of 30 μg.ml⁻¹ of FH₄ to folate-deficient marrows (mean 203% of control, Fig. 2) and (³H)-dUMP accumulation was reduced to a mean of 13.5% (Fig. 3). In neither test was FH₄ as effective as formyl-FH₄.

Methyl-Tetrahydrofolate (Methyl-FH₄)

Addition of 30 μg.ml⁻¹ of this compound to vitamins B₁₂-deficient marrows did not correct the dU suppression test (Fig. 1) mean 14 (± 7.6)%). Concentrations as high as 300 μg.ml⁻¹ did not restore a normal dU suppression value (Table 1). Nevertheless, (³H)-dU incorporation was slightly increased to a mean of 147 (±66)% of control (Fig. 2), while (³H)-dUMP accumulation was decreased slightly to a mean of 95 (±22)% (Fig. 3). Thus, by all three criteria, methyl-FH₄ was much less effective than either formyl-FH₄ or FH₄ in correcting the abnormality in vitamin B₁₂-deficient marrows (see also Fig. 4).

However, 30 μg.ml⁻¹ of methyl-FH₄ reduced the mean dU suppression test value of folate-deficient marrows from 15.8 (±4.5)% to 4.5 (±1.2)% (Fig. 1). Correction was obtained even at 3 μg.ml⁻¹ of methyl-FH₄ (Table 1). (³H)-dU labeling of folate deficient marrows was also increased (mean 200%, Fig. 2) and (³H)-dUMP accumulation reduced at 19% (Fig. 3).
C

Concentration (pg/ml)

30

Fig. 4. DNA labeling and accumulation of labelled (3H)-dUMP in bone marrow cells from a patient with megaloblastic anemia due to vitamin B12 deficiency: effect of varying concentrations of hydroxobalamin or of various folate analogues. Addition of (●), formyl-FH4 (■), FH4 (▲), methyl FH4 or (□), hydroxocobalamin. Solid line incorporation of (3H)-dU into DNA, dashed lines, accumulation of (3H)-dUMP. Data is presented as a percent of the control value in the absence of added vitamins.

Folic (Pteroylglutamic) Acid

Folic acid (50 µg.ml⁻¹) restored a normal dU suppression (mean 3.1%, Fig. 1) to vitamin B12-deficient marrows. Only a small correction was obtained at 5 µg.ml⁻¹ and there was no correction at all at 1 µg.ml⁻¹ (Table 1). (3H)-dU incorporation was increased to a mean of 285% (Fig. 2) and (3H)-dUMP accumulation reduced to a mean of 49% (Fig. 3).

In a single case of folate deficiency tested, 50 µg.ml⁻¹ of folic acid corrected the abnormal dU suppression to a value of 2.3% (Fig. 1). The correction was also observed at 5 µg.ml⁻¹ folic acid, but not at 1 µg.ml⁻¹ (Table 1).

DISCUSSION

In this study we have investigated the ability of vitamin B12 (hydroxocobalamin) and of various folate analogues to overcome the block in thymidylate synthesis in megaloblastic anemia. Three in vitro tests were used: (1) the indirect dU suppression test, (2) the incorporation of (3H)-dU into DNA and (3) the accumulation of (3H) dUMP derived from (3H)-dU in megaloblastic marrows. The methods correlated well: where the dU suppression test value was increased, the accumulation of (3H)-dUMP was increased compared to normal. The absolute incorporation of (3H)-dU into DNA was similar to normal in untreated cells but much higher than normal on addition of the appropriate vitamin. This may be due to the far greater number of proliferating primitive cells in the megaloblastic compared to normal marrow.

The results on corrections of these abnormalities in megaloblastic marrows can be summarized as follows: In all types of megaloblastic anemia, formyl–FH4 was most effective in overcoming the lesion in DNA precursor synthesis. In megaloblastic anemia due to vitamin B12 deficiency, vitamin B12 itself was also effective and both FH4 and folic acid were effective, though less so than formyl–FH4. On the other hand, methyl–FH4 gave very poor (and not significant) correction in vitamin B12 deficiency.

In some experiments, the effects of added folate compounds on vitamin B12-deficient marrows were tested at varying concentrations. In the dU suppression test formyl–FH4 gave excellent correction at levels as low as 0.3 µg.ml⁻¹, while FH4 only gave a correction above 6 µg.ml⁻¹. In contrast, methyl–FH4 did not restore normal dU suppression in vitamin B12 deficiency even at 300 µg.ml⁻¹. The same order of efficacy of these compounds (formyl–FH4 > FH4 > methyl FH4) was also revealed by their titration in the (3H)–dU incorporation test.

Vitamin B12 was ineffective in folate deficiency. As in vitamin B12 deficiency, FH4 and folic acid were effective, though less so than formyl–FH4. Most important, methyl–FH4 overcame the block in thymidylate synthesis in all of the folate deficient cases studied providing evidence for the stability of this compound under our conditions of use. This is in contrast to the results of Deacon et al., who found that treatment with 30 µg.ml⁻¹ methyl–FH4 did not restore normal dU suppression in folate deficiency. This discrepancy may be due in part to technical differences. Methyl–FH4, which is unstable, must be prepared freshly for each study. Another difference in technique between this work and that of Deacon et al., is our use of 1 mM dU in the suppression test, three times the amount used by Deacon et al. We find that the discrimination between normal and borderline cases is thus greatly improved (K. Ganeshaguru & A. V. Hoffbrand, unpublished). Our findings with methyl–FH4 in the dU suppression test were supported by a similar pattern of correction by methyl–FH4 in the (3H)–dU incorporation studies.

There are currently two main hypotheses of how vitamin B12 deficiency results in impaired DNA pre-
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cursor synthesis and hence in megaloblastic anemia. Vitamin B<sub>12</sub> is required as a coenzyme in the reaction catalyzed by methionine synthase (E.C. 2.1.1.13), in which the methyl group of methyl–FH<sub>4</sub> is transferred to homocysteine, yielding methionine and FH<sub>4</sub>. According to the original methyl tetrahydrofolate 'trap' hypothesis, methyl–FH<sub>4</sub> is a "dead" compound and FH<sub>4</sub> is needed to form other folate coenzymes inside cells.\(^{16}\) More recently, it has been proposed that methyl–FH<sub>4</sub> is not a substrate for the synthesis of the polyglutamate forms of folate which are required as coenzymes in intracellular reactions, including the thymidylate synthase step which requires 5,10-methylen–FH<sub>4</sub> polyglutamate as a cofactor.\(^{20}\)

According to the "formate starvation hypothesis"\(^{17,21}\), the emphasis is shifted from B<sub>12</sub>–dependent generation of FH<sub>4</sub> to the B<sub>12</sub>–dependent provision of formate via methionine. The formate is presumed to be required to synthesize formyl FH<sub>4</sub>, which Chanarin et al.\(^{21}\) suggest may be the major substrate for folate polyglutamate synthesis. This view is supported by experiments in which polyglutamate synthesis from various forms of radioactive FH<sub>4</sub> coenzymes were measured in rats in which a functional vitamin B<sub>12</sub> deficiency had been induced by exposure in nitrous oxide.\(^{21}\) Formyl FH<sub>4</sub> but not FH<sub>4</sub> itself proved to be capable of acting as a substrate for polyglutamate synthesis after B<sub>12</sub> inactivation. In support of this hypothesis, Deacon et al.\(^{17}\) found that formyl–FH<sub>4</sub> but not FH<sub>4</sub> could correct the dU suppression test in human vitamin B<sub>12</sub> deficiency. An additional importance of formate supply could be in the provision of the single carbon unit transferred to dUMP from 5,10-methylene FH<sub>4</sub>.

The findings here that FH<sub>4</sub> and folic acid itself are effective in overcoming the lesion in vitamin B<sub>12</sub> deficiency studied in three different ways while methyl–FH<sub>4</sub> is not is compatible with the methyl tetrahydrofolate trap hypothesis. Our results with titrated concentrations of the various folate compounds do confirm, however, that formyl–FH<sub>4</sub> is more effective than FH<sub>4</sub> at correction in vitamin B<sub>12</sub> deficiency. This may support a secondary role of vitamin B<sub>12</sub> in provision of formate via methionine synthesised in the vitamin B<sub>12</sub>–dependent synthase reaction. However, the fact that formyl–FH<sub>4</sub> is more stable than FH<sub>4</sub> and that formyl–FH<sub>4</sub> was in our studies more active than FH<sub>4</sub> even in folate deficiency makes it difficult to interpret whether the difference in activity of FH<sub>4</sub> and formyl–FH<sub>4</sub> is one of stability or membrane transport rather than in the generation of formate. The substantial correction with FH<sub>4</sub> suggests that provision of this compound is at least partly rate limiting for thymidylate synthesis in vitamin B<sub>12</sub> deficiency. It has recently been reported that dihydrofolate is a superior substrate for polyglutamate synthesis by mouse liver folate polyglutamate synthase to FH<sub>4</sub> or formyl–FH<sub>4</sub>, and that the latter two compounds are of approximately equal activity.\(^{24}\)

The controversy over the interaction of vitamin B<sub>12</sub> and folate metabolism remains unresolved. However, it appears to us that vitamin B<sub>12</sub>–dependent demethylation of methyl–FH<sub>4</sub> and vitamin B<sub>12</sub>–dependent provision of formate for synthesis of formyl–FH<sub>4</sub> are both potential rate-limiting steps in folate polyglutamate synthesis. The results here suggest that it is in the provision of FH<sub>4</sub> from methyl–FH<sub>4</sub> that is the main lesion in vitamin B<sub>12</sub> deficiency, although an additional lesion in the provision of formate cannot be excluded.

Finally, our results provide added confirmation of the agreement in diagnosis of vitamin B<sub>12</sub> or of folate deficiency by the serum B<sub>12</sub> or folate assays on the one hand and by the dU suppression test on the other. The \((^{3}H)\)-dU incorporation reactions studied here are also in complete accord with diagnosis by the more established methods.

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