Defective DNA Synthesis in Human Megaloblastic Bone Marrow: Effects of Homocysteine and Methionine

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ABSTRACT In B_{12} deficiency, inadequate DNA synthesis seems due in large measure to a block of tetrahydrofolic acid (THFA) regeneration from 5-methyl THFA (via homocysteine transmethylation).

In support of the above, homocysteine appears to facilitate and methionine to reduce de novo DNA synthesis. This was measured by the ability of deoxyuridine to suppress thymidine-^{3}H uptake into DNA in human bone marrow cultures. The homocysteine effect in B_{12}-deficient marrow supports the possibility that there is in man an additional B_{12}-independent pathway for regeneration of THFA by methylation of homocysteine to form methionine.

Among possible explanations for the methionine effect is end-product inhibition of the homocysteine transmethylase reaction, resulting in further accumulation of 5-methyl THFA. Homocysteine transmethylation may play an important role in the regulation of THFA availability and de novo DNA synthesis.

In vitro and in vivo evidence suggests that methionine may be useful to potentiate and homocysteine to reduce methotrexate action.

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INTRODUCTION

Defective de novo DNA synthesis, as measured by reduced incorporation of deoxyuridine (dU) into the thymine of DNA, has been found in bone marrow from patients with B_{12}- or folate-deficient megaloblastic anemia (1, 2). It was demonstrated (2) that this defect was partially corrected by added B_{12} in the B_{12}-deficient but not the folate-deficient marrows and completely corrected by pteroylglutamic acid (PGA) in both types of deficient marrows. The corrective effect of B_{12} was blocked by methotrexate (MTX) (a folate antagonist). 5-methyltetrahydrofolic acid (THFA), which may accumulate in B_{12} deficiency (3, 4), failed to correct the defect in DNA synthesis in B_{12}-deficient marrows (2), unless B_{12} was added to the culture system.[1] The conversion of 5-methyl THFA to THFA via homocysteine transmethylation is dependent on a B_{12} enzyme (5–7). These findings support the concept that inadequate DNA synthesis in B_{12} deficiency is due in large measure to reduced 5-methyl folate utilization brought about by lack of B_{12} (Fig. 1).

In the present study, the effect of homocysteine (substrate) and methionine (product) on the homocysteine transmethylation step was studied (8). Addition of homocysteine would be expected to release accumulated 5-methyl THFA and increase the THFA available for de novo thymidylate (dTMP) synthesis. Conversely, methionine would be expected to decrease available THFA.

METHODS

Effective de novo synthesis of dTMP from dU in human bone marrow was measured by the ability of 1 hr preincubation with dU to suppress incorporation into DNA of subsequently added thymidine-^{3}H (TdR-^{3}H) as previously described by Killman (1) and modified by Metz et al. (2).

[1] Waxman, S., J. Metz, and V. Herbert. Unpublished data.

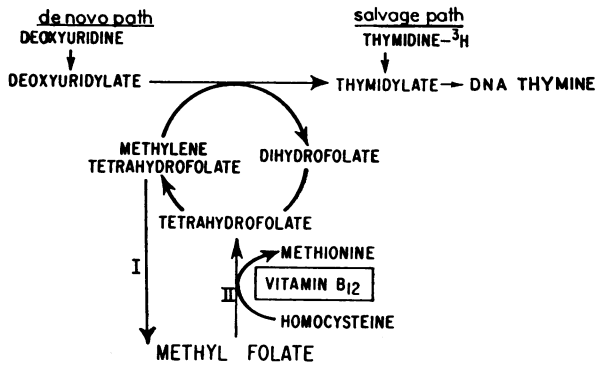

FIGURE 1 B_{12}, folic acid, methionine, and homocysteine interrelationships in thymidylate synthesis. I, 5,10-methylenetetrahydrofolic reductase; II, homocysteine transmethylase.

In this system abnormal de novo DNA synthesis in megaloblastic anemia was demonstrable by reduced ability of preincubation (at room temperature for 1 hr) with dU to suppress incorporation of subsequently added TdR-^{3}H (1 μc/tube). For culture, 15–20 ml of marrow was aspirated into a syringe containing 10 ml of cold Hank's solution with heparin (10,000 U). All operations were carried out as previously described by Metz et al. (2). The radioactive precursor used was methyl thymidine-^{3}H (specific activity 12.5 c/mmole/liter), prepared as a solution containing 10 μc/ml with a concentration during the incubation experiments of 0.134 mμmole/ml. After 3 hr incubation at 37°C, RNA and DNA were extracted from the lysate by the technic described by Feinendegen, Bond, and Painter (9) as modified by Cooper and Rubin (10). The radioactivity of the RNA and DNA extracts was measured in a Picker liquid scintillation counter (Picker Nuclear, New York), and results were expressed as total radioactivity incorporated into DNA.

At the time of aspiration of the bone marrow samples, venous blood was assayed for serum B_{12} (11) and folate (12).

Patients studied. 11 patients with megaloblastic bone marrow changes were studied. Five patients (Nos. 1–5) had B_{12} deficiency, 3 patients (Nos. 6–8) had nutritional folate deficiency, and 3 patients (Nos. 9–11) had MTX-induced folate deficiency. The serum vitamin levels, degree of megaloblastic change in the marrow, and dU suppression effect are shown in Table I. Control subjects (patient Nos. 12–16) were

TABLE I
Summary of the Patients Studied

Patient No.	Diagnosis	Serum vitamin B_{12}	Serum folate	Bone marrow*	dU Suppression of TdR-^{3}H into DNA
		pg/ml	*ng/ml*		%
B_{12} deficient					
1	Pernicious anemia	0	9.5	Marked	38
2	Pernicious anemia + Fe deficiency	81	7	Marked	40
3	Pernicious anemia	0	3.8	Marked	52
4	Pernicious anemia	79	18.5	Marked	55
5	Ileitis	61	17.2	Marked	45
Folate deficient					
6	Macrocytic anemia of pregnancy	171	2.3	Mild	15
7	Alcoholic combined nutritional anemia	737	1.7	Mild	19
8	Nutritional megaloblastic anemia	353	<1	Moderate	37
9	Methotrexate-treated Kaposi's sarcoma	2508	<1	Moderate	33
10	Methotrexate-treated acute leukemia in remission	201	<1	Moderate	29
11‡	Methotrexate-treated Darier's disease	287	<1	Normoblastic	15
Normal					
12	Normal	751	9.5	Normoblastic	8
13	Alcoholic	930	3.4	Normoblastic	7
14	Normal	260	6.5	Normoblastic	3
15	Normal	784	5.8	Normoblastic	4
16	Alcoholic	657	3.9	Normoblastic	6

TdR, thymidine. In our laboratory, serum vitamin B_{12} levels below 100 pg/ml and folate levels below 3 ng/ml indicate unequivocal deficiency. The lower limit for unequivocally normal serum vitamin B_{12} level is 200 pg/ml and for folate, 7 ng/ml. Serum folate above 24 ng/ml is above our upper limit of normal.

* Degree of megaloblastosis.

‡ Studied on several occasions (Fig. 6).

nonanemic hospital patients with normoblastic marrows and normal serum B_{12} and folate levels.

RESULTS

Effect of homocysteine and methionine on dU suppression of TdR-^{3}H incorporation into DNA in normal marrow. In normal marrow the dU enters the deoxyuridylate (dUMP)—dTMP—thymine of DNA pathway, so that the incorporation of TdR-^{3}H is diminished. The degree of diminution (suppression) of TdR-^{3}H uptake is thus a measure of dUMP incorporated into DNA. In normal marrow, TdR-^{3}H uptake was diminished to 10% or less of control values (i.e., samples preincubated without added dU) when the marrow was preincubated with dU 10^{-1} μmole/tube (Fig. 2), as previously reported (2). This diminution (suppression) implies normal DNA synthesis. The addition of L-homocysteine (1 mg/tube) with dU had little effect on the normal dU-suppressive effect on TdR-^{3}H uptake into DNA. L-Methionine (1 mg/tube) reduced the dU effect ($P < 0.01$), most dramatically in patients 13 and 16 who were alcoholics with low-normal serum folate level. PGA (50 μg/tube), when added with methionine and dU, returned the dU effect to normal range.

Effect of homocysteine and methionine on dU suppression of TdR-^{3}H incorporation into DNA in B_{12}-deficient marrow. The subnormal dU suppression of TdR-^{3}H incorporation in B_{12}-deficient marrow was partially corrected by L-homocysteine (1 mg/tube) when added with dU in the absence of B_{12} ($P < 0.02$). Homocysteine added with B_{12} (1 μg/tube) enhanced the B_{12} correction ($P < 0.02$) (Fig. 3). In one B_{12}-deficient marrow (patient No. 5) homocysteine with dU corrected the dU suppression of TdR-^{3}H from 5377 cpm (45%) to 2876 cpm (24%), and this homocysteine effect was not significantly altered in the presence of B_{12} anilide[2] (0.1 mg) (a B_{12} antagonist), 2374 cpm (20%). Conversely, the corrective effect of B_{12} was blocked by L-methionine (1 mg) ($P < 0.05$). Methionine, when added together with dU, did not alter significantly the defective dU suppression of TdR-^{3}H in B_{12}-deficient marrow.

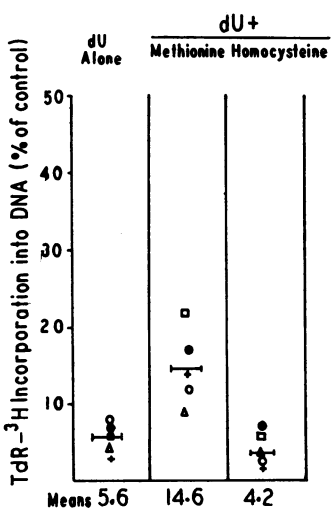

FIGURE 2 Effect of homocysteine and methionine on deoxyuridine (dU) suppression of TdR-^{3}H incorporation into DNA in normal marrow. Patient Nos.: 12 (○), 13 (●), 14 (+), 15 (△), 16 (□); mean (|—|); dU, deoxyuridine 10^{-1} μmole, TdR-^{3}H, thymidine-^{3}H, 1 mg of L-methionine and 1 mg of L-homocysteine added.

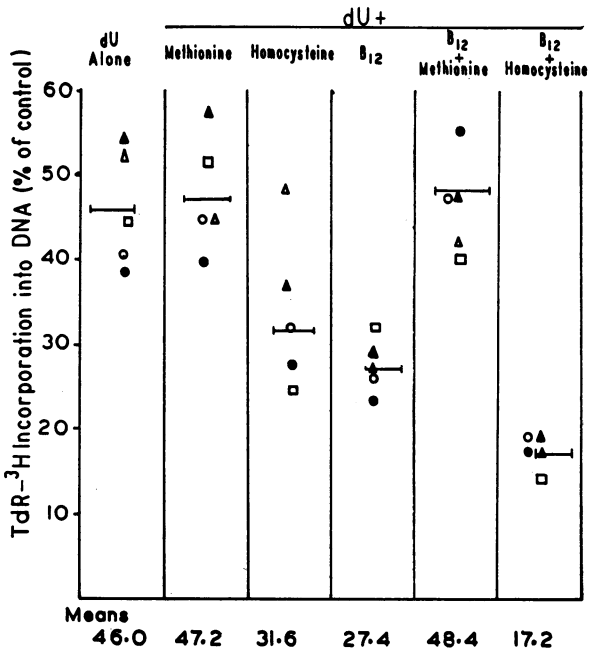

FIGURE 3 Effect of homocysteine and methionine on dU suppression of TdR-^{3}H incorporation into DNA in B_{12}-deficient marrow. Patient Nos.: 1 (●), 2 (○), 3 (△), 4 (▲), 5 (□); mean (|—|); dU, deoxyuridine 10^{-1} μmole, TdR-^{3}H, thymidine-^{3}H, 1 mg of L-methionine, 1 mg of L-homocysteine, and 1 μg of B_{12} added.

Effect of homocysteine and methionine on dU suppression of TDR-^{3}H incorporation into DNA in folate-deficient marrow. The subnormal dU suppression of TdR-^{3}H in folate-deficient marrow was not significantly affected by the addition of L-homocysteine to the dU (Fig. 4). L-Methionine further impaired dU suppression of TdR-^{3}H incorporation into DNA ($P < 0.05$). The severity of impairment appeared to correlate well with degree of folate deficiency and megaloblastic change in the marrow.

Effect of homocysteine and methionine on MTX-induced defective dU suppression of TdR-^{3}H. MTX (1

[2] Vitamin B_{12} anilide was generously provided by Dr. E. Lester Smith (now retired), Glaxo Laboratories, Greenford, England.

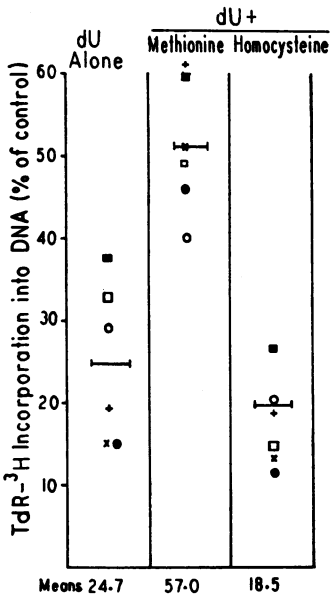

FIGURE 4 Effect of homocysteine and methionine on dU suppression of TdR-^{3}H incorporation into DNA in folate-deficient marrow. Patient Nos.: 6 (×), 7 (+), 8 (■), 9 (□), 10 (○), 11 (●); mean (|—|); dU, deoxyuridine 10^{-1} μmole, TdR-^{3}H, thymidine-^{3}H, 1 mg of L-methionine and 1 mg of L-homocysteine added.

μmole) added with dU to three normal marrow cultures markedly interfered with normal dU suppression of TdR-^{3}H incorporation into DNA ($P > 0.02$) (Fig. 5). L-Methionine (1 mg) incubated for 10 min in the culture before the addition of dU and MTX further impaired dU effect beyond the impairment produced by

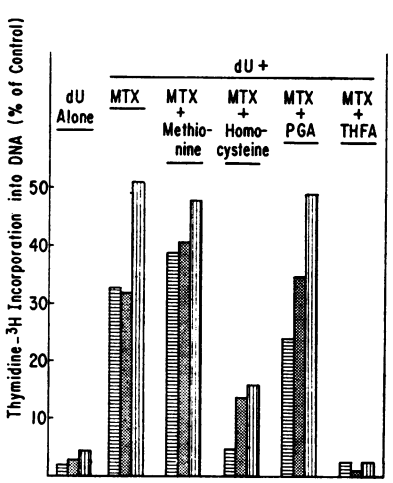

FIGURE 5 In vitro methotrexate effect on dU suppression of TdR-^{3}H incorporation into DNA. dU, deoxyuridine 10^{-1} μmole, TdR-^{3}H, thymidine-^{3}H; MTX, methotrexate 10^{-6} mole/liter; L-methionine, 1 mg; L-homocysteine, 1 mg; PGA, pteroylglutamic acid; THFA, tetrahydrofolic acid, folinic acid. Similar shaded bars represent the same patient.

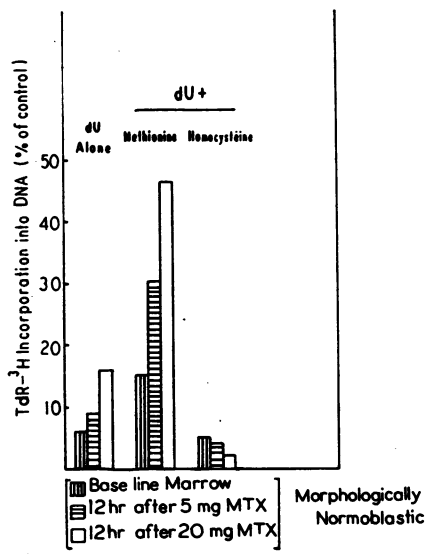

FIGURE 6 In vivo methotrexate effect on dU suppression of TdR-^{3}H incorporation into DNA. dU, deoxyuridine 10^{-1} μmole, MTX, methotrexate. 1 mg of L-homocysteine and 1 mg of L-methionine added.

MTX alone. However, if MTX was added to the culture before methionine no further impairment of dU suppression of TdR-^{3}H by methionine was demonstrated. The addition of L-homocysteine partially corrected the effect of MTX. PGA added in amounts by weight up to 1650 times that of MTX failed to completely correct the MTX effect, whereas reduced folate (folinic acid), in the form of leucovorin, by weight 25 times that of MTX, corrected dU effect to normal.

Serial marrows were obtained from a patient (No. 11) receiving MTX for Darier's disease (Fig. 6). Before MTX therapy, dU suppressed TdR-^{3}H incorporation into DNA in normal fashion. Marrows, although morphologically normoblastic 12 hr after the patient received 5 mg and then 20 mg of MTX, demonstrated nevertheless inability of dU to suppress TdR-^{3}H into DNA. This effect was greater after the larger dose of MTX. When added in vitro, methionine enhanced MTX effect in direct relationship to the amount of MTX taken by the patient. Homocysteine, when added in vitro, appeared to correct the defective dU effect produced by in vivo MTX.

DISCUSSION

dTMP for DNA synthesis (13) can arise either via the salvage pathway, from preformed TdR (14), or via the de novo pathway, from dUMP (Fig. 1) (15). In the de novo sequence, the one-carbon unit is reduced and transferred from 5,10-methylene THFA to dUMP. This process is accompanied by the oxidation of THFA to dihydrofolic acid (DHFA) (16). Thus, in order for

de novo dTMP synthesis to proceed, THFA must be continuously available and acquire a one-carbon unit to become 5,10-methylene THFA. THFA availability may be reduced by nutritional folate deficiency, folic acid antagonists, or by interference with the homocysteine-methionine transmethylation pathway (which depends on a B_{12} enzyme), whose main function in man may be to regenerate THFA from 5-methyl THFA.

In vitro evidence previously reported (2) suggests that subnormal synthesis of the thymine of DNA in B_{12}-deficient megaloblastic bone marrow is due in significant measure to deranged folate metabolism associated with the B_{12} deficiency. Herbert and Zalusky (3) showed that *L. casei*-active material, subsequently identified as 5-methyl THFA (4), accumulated in the serum in B_{12} deficiency. Since the conversion of 5-methyl THFA via homocysteine transmethylation to THFA requires B_{12} as coenzyme (5–7), in B_{12}-deficiency states 5-methyl THFA may accumulate, reducing the pool of available THFA. Among other consequences, such a pool reduction would result in less 5,10-methylene THFA available for coenzyme functions, such as the methylation of dUMP to dTMP.

In the system utilized in the current studies, reduced de novo DNA synthesis in megaloblastic bone marrow appeared to be due to interference with the methylation of dUMP to dTMP, which is unequivocally a folate-dependent step (17–24). Homocysteine enhanced B_{12} correction of the defective de novo dTMP synthesis in B_{12}-deficient marrow, perhaps by releasing stores of 5-methyl THFA for regeneration of THFA. Correction by homocysteine of the reduced dU suppression of TdR-^{3}H in either the absence of B_{12} or in the presence of B_{12} anilide (a B_{12} antagonist) in B_{12}-deficient marrow may support the possibility in man of an *additional, B_{12}-independent pathway* for regeneration of THFA by methylation of homocysteine to form methionine, as has been suggested by the preliminary studies of Foster in man (25) and as Kisliuk and Woods found in bacteria (26).

However, there are alternate possibilities: (*a*) the B_{12}-dependent pathway may be increased by large amounts of homocysteine; or (*b*) in the presence of large amounts of homocysteine, methionine may be formed nonenzymatically by mass action; or (*c*) the homocysteine effect may be indirect and via an as yet unrecognized mechanism. Homocysteine had little effect in folate-deficient marrows. This finding is consistent with absence of accumulation of 5-methyl THFA, owing to the fact that homocysteine transmethylation is not obstructed as in states of B_{12} deficiency.

Among possible explanations for the apparent interference by methionine with de novo dTMP synthesis in normal, folate-deficient, and B_{12}-deficient marrow is end-product inhibition of the homocysteine transmethylase reaction, resulting in accumulation of 5-methyl THFA. This result is similar to the result of methionine repression of homocysteine transmethylase, as demonstrated by Dickerman, Bieri, Redfield, and Weissbach (27) in chickens and Kutzback, Galloway, and Stokstad (28) in rats. The methionine effect in the present study appeared to be related to the availability of THFA, because the most marked effect in normal marrows occurred in two alcoholic patients with low-normal folate levels, and the methionine effect could be corrected by the addition of PGA.

Methionine has been reported to aggravate clinical B_{12}-deficient megaloblastic anemia (29) and also to decrease formiminoglutamic acid urinary excretion (30–32). Katzen (33) has demonstrated that the synthesis of 5,10-methylene THFA reductase is severely repressed by methionine in bacteria, but Kutzback et al. (28) found no such repression in rat liver by methionine. Methionine appears to have a regulatory role via the transmethylation reaction in the tissue distribution of folic acid coenzymes. The importance in man of homocysteine transmethylation may be to regenerate THFA rather than to generate methionine, since methionine is abundantly available in the diet.

The effect of varying amounts of MTX in vitro and in vivo was measured by the dU suppression of TdR-^{3}H uptake into DNA. Reduced dU suppression of TdR-^{3}H was exaggerated as a patient received more MTX and was apparent before any morphologic evidence of megaloblastosis was evident. In vitro studies revealed that MTX effect could be enhanced by methionine, perhaps by blocking THFA regeneration from 5-methyl THFA. Such an effect would be additive to the MTX ability to block THFA production from PGA and DHFA by binding dihydrofolate reductase (34–37). Homocysteine appeared to correct MTX effect in vitro, perhaps by releasing intracellular stores of 5-methyl THFA for THFA regeneration, thus by-passing the blocked DHFA. The efficacy of methionine to potentiate and homocysteine to reduce MTX action in vivo is now under study.

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