THE EFFECT OF HOMOCYSTEINE, METHIONINE, SERINE AND GLYCINE ON DNA SYNTHESIS BY HUMAN NORMOBLASTIC AND MEGALOBLASTIC BONE MARROW CELLS

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Summary Both glycine and methionine, when added to a suspension of human bone marrow cells, impaired the utilization of deoxyuridine for DNA synthesis, using either the uptake of 3H-deoxyuridine or the subsequent uptake of 3H-thymidine as an index. Homocysteine reduced the uptake of both 3H-deoxyuridine and 3H-thymidine, indicating interference with DNA synthesis after the stage of thymidylate synthesis. Another explanation that the decreased uptake of both substances by homocysteine was due to cell damage caused in vitro was suggested by the trypan blue viability test. Serine generally did not produce significant effects. No difference could be detected between the results in normoblastic and megaloblastic marrow.

The deoxyuridine suppression (DUS) test introduced by Killman (1) and Metz et al. (2) measures the conversion of deoxyuridine (UdR) to deoxythymidine usually in bone marrow cells. This step requires functioning folate coenzymes and hence is abnormal in vitamin B12 and folate deficiency associated with megaloblastic anemia.

Amino acids that are concerned with single carbon unit metabolism influence folate metabolism (Fig. 1). In vivo methionine (Met) administration will reduce formiminoglutamic acid excretion in untreated pernicious anemia and in a variety of experimental animals. In addition, Met alleviates the effects of vitamin B12 deficiency in rats and this aspect has been reviewed by Shin et al. (3). The effect of homocysteine (Homo) and Met on the DUS test has been reported by Waxman et al. (4) and Cheng et al. (5).

The methylfolate trap hypothesis (Herbert and Zalusky (6), Noronha and
Fig. 1. Pathways relating to metabolism of folate and amino acids. THF, tetrahydrofolic acid; UdR, deoxyuridine; TdR, thymidine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; Ser, serine; Gly, glycine; Met, methionine; Homo, homocysteine.

Silverman (7) suggests that there are two folate pools in the body, a methyltetrahydrofolate pool and tetrahydrofolate pool, and entry from the first to the second pool requires vitamin B12 acting via Homo methyl-transferase in the Homo-Met reaction. In megaloblastic anemia caused by vitamin B12 deficiency, the methylfolate pool expands at the expense of the tetrahydrofolate pool since metabolism of methyltetrahydrofolate is impeded because of vitamin B12 lack.

The purpose of this study was threefold, viz., to see if the results of the direct uptake of 3H-UdR by marrow paralleled the results obtained in the DUS test, to see the effects of the addition of amino acids giving or receiving single carbon units such as glycine (Gly), serine (Ser), Homo and Met, and thirdly to see if there were any significant differences in the behavior of normoblastic vs megaloblastic marrows in these tests; that is, did marrow with one or two folate pools behave differently.

MATERIALS AND METHODS

The DUS test was performed by the method described by Metz et al. (2). Three to 5 ml of bone marrow aspirate was mixed with 10 ml of Hanks balanced salt solution (HBSS), pH 7.4, containing 100 IU/ml of preservative free heparin. All reagents were dissolved in HBSS and pH was adjusted to 7.4. After washing in HBSS three times, bone marrow cells were suspended in autologous serum–HBSS (1:3) and cell counts were adjusted to 2–12x10^8/ml. Assay mixtures contained marrow cells (0.3 ml) plus UdR (0.1 μmol), with addition of either L-Met (1 mg), Homo (1 mg), d-L-Ser (2 mg) or Gly (1 mg) in a total volume of 0.9 ml. Controls were set up with HBSS replacing amino acids. All tests were set up in duplicate or triplicate. The mixture were incubated at 37°C for 1 hr shaking gently (90 strokes/min) on a metabolic shaker. After the incubation 3H-thymidine (Tdr) (specific activity; 23–25.6 Ci/mmol) or 3H-UdR (specific
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activity; 21–27 Ci/mmol) purchased from The Radiochemical Centre (Amersham, England) and prepared as a solution containing 10 μCi/ml, were added to each tube in a volume of 0.1 ml. The marrow cells were incubated further for 3 hr at 37°C. DNA was extracted by the method of Feinidegen et al. (8) modified by Cooper and Rubin (9). Radioactivity was measured by adding 0.2 ml of DNA extracts to 18 ml of scintillation fluid [toluene with 30% methanol containing 0.3% of 2,5-diphenyloxazole and 0.03% p-bis(2-(5-phenyloxazole))-benzene, modified from Gione et al. (10)] and measuring with a Wallac-LKB liquid scintillation counter. Results were expressed as the percentage of the dpm of ³H-TdR or ³H-UdR incorporated into DNA.

Effects of Homo and Met were studied using 15 normoblastic and 6 megaloblastic marrows. Effects of Ser and Gly were examined using 13 normoblastic and 9 megaloblastic marrows. All the patients were undergoing marrow aspiration as part of their normal clinical investigation. Ten out of twelve patients with megaloblastic anemia had pernicious anemia and the remaining two folate deficiency. There was no difference in the results obtained in the folate or vitamin B₁₂ deficient patients and hence the results in the megaloblastic patients have been pooled.

The trypan blue viability test on the cells from 4 normoblastic marrows was performed as described by Bazerbash (11) after the incubation with either L-Met (1 mg), D-L-Homo (2 mg), D-L-Ser (2 mg) or Gly (1 mg) for 1 hr. One volume of cell suspension was mixed with an equal volume of 0.4% trypan blue in HBSS with 5% foetal calf serum and left at room temperature for 10 min. The number of non-viable cells taking up the trypan blue were counted in a counting chamber.

RESULTS

The addition of Homo to marrow cells impaired the incorporation of TdR into DNA as well as the incorporation of UdR (Fig. 2). As the late stage of DNA synthesis as assessed by impaired utilization of TdR is interfered with, it is not possible with the methods used to know whether there was any effect on earlier stages.

The overall effects of the addition of Gly and Met were similar. Both reduced the incorporation of UdR into DNA when measured directly or indirectly by the DUS test (Figs. 2 and 3). Conversely, there was an increase in TdR uptake above control levels with these amino acids which was statistically significant with Met with both normoblastic and megaloblastic marrows, but only significant with the megaloblastic group with Gly.

Ser did not have any effect on UdR or TdR uptake, nor was there any effect on the DUS test with megaloblastic marrows (Fig. 2). With the control group, the % “suppression” was 5% as compared with 6.7% in the absence of Ser, a difference that was significant with the t test. The pattern of response of the
Fig. 2. Effect of homocysteine and methionine on deoxyuridine suppression test and incorporation of \(^{3}H\)-thymidine and deoxyuridine. DUS, deoxyuridine suppression; Normo, normoblastic marrows; Megalo, megaloblastic marrows. Other abbreviations are as shown in Fig. 1.

\[ * p < 0.05 \quad ** p < 0.01 \quad *** p < 0.001 \]

Fig. 3. Effect of serine and glycine on deoxyuridine suppression test and incorporation of \(^{3}H\)-thymidine and \(^{3}H\)-deoxyuridine. NS, non-significant. Abbreviations are as shown in Figs. 1 and 2.

\[ * p < 0.05 \quad ** p < 0.01 \quad *** p < 0.001 \]

normoblastic and megaloblastic group was the same.

The trypan blue viability test on the bone marrow cells incubated with various amino acids showed significant increase of non-viable cells only in the cells incubated with Homo (Table 1).

**DISCUSSION**

The effects of Met on vitamin B\(_{12}\) and folate metabolism have been extensively
Table 1. Trypan blue viability test on 4 normoblastic marrows after incubation with various amino acids.

<table>
<thead>
<tr>
<th></th>
<th>Means %</th>
<th>Standard deviations</th>
<th>t test</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.5</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>9.8</td>
<td>3.86</td>
<td>p&lt;0.01</td>
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<td>Methionine</td>
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<td>0.82</td>
<td>non-significant</td>
</tr>
<tr>
<td>Serine</td>
<td>3.0</td>
<td>0.82</td>
<td>non-significant</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.3</td>
<td>0.50</td>
<td>non-significant</td>
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studied in rats and sheep. In the vitamin B₁₂-deficient animals Met tends to overcome the effect of vitamin B₁₂ lack (3, 12–15). The relevance of these observations to man however remains uncertain although a single report of RUNDLES and BREWER (16) indicated that Met aggravated megaloblastosis in the bone marrow of vitamin B₁₂-deficient patients. This contrary action observed in vivo was confirmed by WAXMAN et al. (4) in vitro. CHENG et al. (5) also demonstrated the antifolate effect of Met in the bone marrow of the vitamin B₁₂-deficient rats with concomitant “profolate effect” of Met in the liver.

In the present study the observations of WAXMAN et al. (4) and CHENG et al. (5) were confirmed. WAXMAN et al. (4) could not show the effect of Met on vitamin B₁₂-deficient marrow but CHENG et al. (5) revealed this effect in vitamin B₁₂-deficient rats. Our result coincided with that of the latter. For the first time in the present study, we observed effects of Met on the incorporation of ³H-UdR and ³H-TdR directly. Met decreased the uptake of UdR and increased that of TdR. This shows that Met actually disturbed the pathway from deoxyuridylate to deoxythymidylate. Theoretically however, the addition of Met should not effect this pathway in megaloblastic marrow cells because it was already suppressed. But the report of WAXMAN et al. (4) showing no effect of Met on DUS test in vitamin B₁₂-deficient marrow was not confirmed by the present study and by that of CHENG et al. (5). No difference was observed in the effect of Met on the normoblastic and megaloblastic marrow. This fact suggests either that the defect of methylation of deoxyuridylate in megaloblastic marrow cells was further aggravated by Met or that the effect of Met should be interpreted as a phenomena appearing only in vitro. Direct measurement of the amount of 5-10-methylene tetrahydrofolic acid available for the methylation of deoxyuridylate is required to solve this question.

WAXMAN et al. (4) also observed the effect of Homo only in vitamin B₁₂-deficient marrow and suggested that Homo facilitated de novo DNA synthesis. Their assumption proved to be incorrect by the present finding that Homo reduced not only the incorporation of UdR but also that of TdR. Also no difference in Homo effect was seen in normoblastic and megaloblastic marrow. Although CHENG et al. (5) supposed that Homo interfered with DNA synthesis at the later levels than deoxyuridylate methylation, another explanation is possible. TISMAN
et al. (17) reported that penicillamine suppressed the incorporation of $^3$H-TdR, $^3$H-UdR and formate-$^{14}$C into DNA of human bone marrow cells and this effect was also seen in other sulf-hydryl containing amino acids, including Homo. We observed that the viability of bone marrow cells was significantly damaged after incubation with Homo though no such effect was observed with Met, Gly or Ser. Therefore, a decreased uptake of UdR and TdR may be due to cell damage by Homo in vitro.

Gly had a similar effect on Met confirming the report of Waxman et al. (18). The most likely explanation may be that the addition of Gly caused the diminution of 5-10-methylene-tetrahydrofolic acid by promoting direction of the reversible reaction by the enzyme Ser hydroxymethyl transferase (Fig. 1) to Ser formation (19). Surprisingly however, Ser, which might be expected to have an opposite effect, produced little change. Although significant change by Ser in the DUS test value in normoblastic marrow was observed, no effect of Ser on TdR or UdR uptake indicates that this finding was rather incidental. Here again further study on the change of the amount of folate coenzymes in the cells is required.

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