Bone Marrow Cells From Vitamin B₁₂- and Folate-Deficient Patients Misincorporate Uracil Into DNA

By S.N. Wickramasinghe and S. Fida

Bone marrow cells from 15 patients with normal deoxyuridine (dU) suppression test results, 3 healthy subjects, and 11 patients with megaloblastic anemia caused by vitamin B₁₂ or folate deficiency were examined for misincorporation of uracil into DNA. Cells were incubated with [5-³H] uridine for 2 hours and their DNA extracted. The DNA was hydrolyzed to deoxyribonucleosides with DNase 1, phospohodiesterase and alkaline phosphatase, and any dU present was separated from other deoxyribonucleosides by Aminex A6 chromatography. The quantity of dU/mg DNA and the radioactivity in the dU peak/mg DNA were then calculated. The results clearly showed that there was markedly increased uracil misincorporation into the DNA of vitamin B₁₂- or folate-deficient marrow cells. Misincorporation of uracil into DNA may be an important biochemical lesion underlying both the megaloblastic change and the ineffectiveness of hematopoiesis in vitamin B₁₂ and folate deficiency.

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METHODS

Marrow samples from three healthy volunteers and 26 patients with various disorders were studied. Freshly aspirated marrow was mixed with heparinized Hanks' solution and the mixture passed sequentially through a 21-ga and a 25-ga needle. The resulting cell suspension was centrifuged and the buffy coat resuspended in tris-buffered Hanks' solution (TBH) to a nucleated cell concentration of 2.5 to 5 x 10⁶/mL. An aliquot of the cell suspension was used for the performance of a dU suppression test using a microtiter plate method. Another aliquot was incubated with 0.5 µCi/mL [5-³H] uridine (specific activity 27 Ci/mmol; Amersham International, Little Chalfont, Bucks, UK) for 2 hours and used for the isolation of DNA. In some experiments, the cells in the remainder of the suspension were resuspended to the same nucleated cell concentration in RPMI 1640 (Imperial Laboratories, Andover, Hants, UK) for 2 hours and used for the isolation of DNA. In some experiments, the cells in the remainder of the suspension were resuspended to the same nucleated cell concentration in RPMI 1640 (Imperial Laboratories, Andover, Hants, UK) for 2 hours and used for the isolation of DNA. In some experiments, the cells in the remainder of the suspension were resuspended to the same nucleated cell concentration in RPMI 1640 (Imperial Laboratories, Andover, Hants, UK) for 2 hours and used for the isolation of DNA.

Isolation of DNA. After washing twice with TBH (pH 7.4), the radiolabeled cells were mixed with 5 mL of lysis buffer (tris-EDTA), pH 7.8, containing 0.5% sodium dodecyl sulfate (SDS); NaCl 150 mmol/L, and 100 µg proteinase K (Sigma) per milliliter. The mixture was incubated at 37°C for 16 hours and extracted with phenol/chloroform (25 vols:24 vols). The aqueous phase was reextracted with chloroform to remove the residual phenol and the aqueous phase incubated at 37°C for 2 hours with 100 µg RNase A Type III (Sigma) per milliliter. One hundred micrograms per milliliter of proteinase K was then added and the mixture incubated for a further 4 to 6 hours. The mixture was again extracted with phenol/chloroform and the aqueous phase reextracted with chloroform.

The DNA was precipitated with 2.5 vol of cold ethanol (~20°C), spun, and cleaned, as described by Saffhill et al. The cleaned DNA was dissolved in water overnight and the total yield calculated.

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from the absorbance at 260 nm, assuming optical density (OD) of 1 is equal to 40 μg of DNA. The purity of DNA was determined from the ratio of absorbance at 260 nm to that at 280 nm, and DNA with a ratio above 1.7 was frozen to await analysis.

**DNA analysis.** One volume of the DNA solution was mixed with an equal volume of digestion buffer (50 mmol/L Thri/HCl, pH 7.4, 5 mmol/L MgCl₂, 3 mmol/L sodium azide, and 1 mmol/L L-2'-deoxycoformycin) and 25 μL/mL of a solution containing 5 mg DNase 1 (Sigma)/mL, after which the mixture was incubated at 37°C for 10 minutes. Snake venom phosphodiesterase (0.3 U/mL) and alkaline phosphatase (3 U/mL) were then added and the mixture was reincubated at 37°C for 5 hours. The resulting hydrolysate was stored frozen until used for chromatography.

Chromatography was performed on a 30 × 1-cm column packed with Aminex A6 (Bio-Rad Laboratories, Richmond, CA) high-perform-ance liquid chromatography (HPLC) grade resin.21 The functional group of the sterene divinyl benzene copolymer was converted to the anion-exclusion state before use, and the column eluted with the ammonium bicarbonate buffer at a flow rate of 18 mL/h, collecting 10-minute fractions, and monitoring the absorbance of the eluate continuously at 254 nm. All the nucleoside peaks were resolved within 30 fractions. The fractions corresponding to each of the deoxyribonucleosides were pooled, the pools were lyophilized and the solids dissolved in 1 mL water. The amount of each nucleoside was determined from an absorbance measurement at the appropriate wavelength applying the extinction coefficients: dU 10², dT 9.6 × 10², deoxycytidine (dC) 9 × 10², deoxyadenosine (dA) 1.5 × 10⁴, and deoxyguanosine (dG) 1.37 × 10⁵; the relevant wavelengths were determined by scanning on a Cecil CE599 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK) between 200 to 320 nm. The radioactivity of each reconstituted nucleoside peak was measured and the total radioactivity per mg DNA calculated.

**Conversion of dC to dU in vitro.** To study the extent of deamination of dC to dU during the isolation of the DNA, 1.5 mg of dC were added to 5 mL lysis buffer and incubated with proteinase K and RNase A Type III as described earlier. At the end of the phenol/chloroform extraction, an aliquot was subjected to Aminex A6 chromatography. Similarly, the extent of deamination during hydrolysis of DNA was studied by mixing 0.3 mg, 0.5 mg, or 1 mg dC in 1 mL of digestion buffer, incubating with DNase, phosphodiesterase, and alkaline phosphatase and analysing an aliquot of the hydrolysate by chromatography.

**RESULTS**

The dU suppression test values (dUSV) in the three healthy volunteers varied between 2.6% and 11.6% and were within the previously established normal range of 2% to 12.8%.19 Fifteen of the patients also showed dUSVs within the normal range, and in these, the eventual diagnoses were lymphoma without marrow infiltration, iron deficiency anemia, alcohol-related macrocytosis, anemia of chronic disorders, or myelodysplastic syndrome. The dUSV in these 15 cases ranged between 1.5% and 12.6%. All 11 cases with an abnormal dUSV had megaloblastic hematopoiesis and the essential clinical and laboratory data in these patients are summarized in Table 1.

The elution profiles obtained after DNA hydrolyses from human bone marrow were applied to the Aminex A6 column showed five deoxyribonucleosides, namely dU, dT, dC, dA, and dG; a dU peak was found in both marrows showing normal and high dUSVs but was larger in the latter (Figs 1 and 2). The elution positions of each of these deoxyribonucleosides were initially identified using pure compounds. Furthermore, added pure dU coeluted with the dU derived from the DNA hydrolysate and added pure uridine did not. The added uridine eluted as a separate peak 5 minutes before the dU peak.

The results of the experiments in which dC was subjected to the same treatment as bone marrow cells showed that there was no deamination of dC to dU during the isolation of the DNA but that about 4% of dC was converted to dU during the hydrolysis of DNA. The results obtained from all bone marrow samples were therefore corrected for this degree of deamination.

The data obtained with the marrow samples from the three healthy volunteers were similar to those from the 15 patients with normal dUSVs, and the data from all 18 subjects were therefore analyzed together. Table 2 and Figs 3 and 4 show the quantity of nucleoside and radioactivity in each nucleoside pool per milligram DNA in the marrows with high and normal dUSVs. DNA hydrolysates from 8 of the 18 individuals with normal dUSVs contained no detectable dU, and hydrolysates from the remaining 10 individuals, including 3 healthy subjects, contained small quantities of dU. In addition, hydrolysates from 13 of 14 individuals with a normal dUSV showed a low level of radioactivity in the fractions corresponding to the dU peak; that from the 14th subject showed no radioactivity in these fractions. By contrast, DNA hydrolysates from patients with a high dUSV showed marked and statistically significant increases both in the quantity of dU derived from DNA and in the radioactivity in the dU per mg DNA. The radioactivity in the dC pool obtained from the DNA of 3 of 10 marrow samples with high dUSVs was greater than the 95% reference limits obtained for marrow samples with normal dUSVs (Table 2 and Fig 4). However, the difference between the geometric means of the radioactivity in the dC pool in the group of patients with high and normal dUSVs was not statistically significant.

As discussed previously,14 the conversion of 3H-uridine into 3H-dUTP would occur via the following pathway: uridine → uridine monophosphate (UMP) → UDP → UTP → CTP → CDP → dCDP → dCMP → dUMP → dUTP. The dU from dUTP would then be incorporated into DNA. The radioactivity in the dT pool would have resulted from a small amount of uridine labeled in the 6 position, which is known to contaminate the [5-3H] uridine used in this study. Following conversion to 3H-dUMP as above, the 3H-dUMP would be methylated to 3H-dTMP and eventually converted to 3H-dTTP, which is the source of dT in DNA.

The reproducibility of the chromatographic technique was studied by performing the procedure in quadruplicate on bone marrow samples with normal and high dUSVs. The coefficients of variation for the quantity of deoxynucleoside per mg DNA in dU, dT, dC, dG, and dA were 8.5%, 2.9%, 5.5%, 9.4%, and 8.2%, respectively, for normoblastic marrows and 7.7%, 2.7%, 7.3%, 6.2%, and 3.3%, respectively, for megaloblastic marrows. The coefficients of variation for
Table 1. Essential Clinical and Laboratory Data on the Patients With Megaloblastic Hematopoiesis

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Hb (g/dL)</th>
<th>MCV (FL)</th>
<th>Serum B$_{12}$ (ng/mL)</th>
<th>Serum Folate (μg/L)</th>
<th>Red Blood Cell Folate (μg/L)</th>
<th>dUSV (%)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHa</td>
<td>M</td>
<td>73</td>
<td>6.2</td>
<td>115</td>
<td>56</td>
<td>445</td>
<td>51.0</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>PHe</td>
<td>M</td>
<td>78</td>
<td>6.3</td>
<td>131</td>
<td>173</td>
<td>69</td>
<td>29.8</td>
<td>Dietary folate deficiency, bronchopneumonia</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>F</td>
<td>60</td>
<td>3.2</td>
<td>151</td>
<td>253</td>
<td>0</td>
<td>40.9</td>
<td>Dietary folate deficiency</td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>F</td>
<td>86</td>
<td>5.9</td>
<td>141</td>
<td>11</td>
<td>73</td>
<td>40.0</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>F</td>
<td>88</td>
<td>7.5</td>
<td>113</td>
<td>37</td>
<td>76</td>
<td>49.5</td>
<td>Dietary B$_{12}$ and folate deficiency</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>F</td>
<td>39</td>
<td>6.7</td>
<td>125</td>
<td>41</td>
<td>267</td>
<td>34.1</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>LF</td>
<td>F</td>
<td>79</td>
<td>7.8</td>
<td>120</td>
<td>23</td>
<td>142</td>
<td>48.3</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>HF</td>
<td>F</td>
<td>61</td>
<td>5.9</td>
<td>117</td>
<td>164</td>
<td>40</td>
<td>32.9</td>
<td>Dietary B$_{12}$ and folate deficiency</td>
<td></td>
</tr>
<tr>
<td>EO</td>
<td>F</td>
<td>65</td>
<td>11.6</td>
<td>105</td>
<td>86</td>
<td>112</td>
<td>28.9</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>JB</td>
<td>F</td>
<td>70</td>
<td>8.9</td>
<td>130</td>
<td>68</td>
<td>296</td>
<td>57.7</td>
<td>Periculous anemia</td>
<td></td>
</tr>
<tr>
<td>MGI</td>
<td>F</td>
<td>75</td>
<td>11.5</td>
<td>117</td>
<td>81</td>
<td>188</td>
<td>49.9</td>
<td>Periculous anemia</td>
<td></td>
</tr>
</tbody>
</table>

Normal ranges: MCV 83 to 99 FL; serum B$_{12}$ 165 to 684 ng/L; serum folate 2 to 8 μg/L; red blood cell folate 200 to 800 μg/L.

The radioactivity per milligram DNA in dU, dT, dC, dG, and dA were similar for marrows with normal and high dUSVs, being 6.8%, 2.3%, 11.0%, 7.3%, and 8.9%, respectively.

When five marrow cell suspensions showing normal dUSVs were incubated without and with MTX for 16 hours, the quantities of dU in hydrolyzed DNA were, respectively, 0.103 (SD 0.070) and 0.116 (SD 0.033) μmol/mg DNA. The corresponding values for radioactivity in the dU pool were 47.6 (SD 39.4) and 180.4 (SD 82.4) cpm/mg DNA. The difference between the control and MTX-treated groups was not statistically significant for the quantity of dU but was significant for the radioactivity data (P < .05; paired t-test). In the experiments in which four preparations of vitamin B$_{12}$- and/or folate-deficient cells were incubated without and with 10 μmol/L thymidine for 16 hours, the quantity of dU in hydrolyzed DNA and the radioactivity in the dU pool were 0.409 (SD 0.101) μmol/mg DNA and 242 (SD 85) cpm/mg DNA for cells not incubated with thymidine, and 0.192 (SD 0.097) μmol/mg DNA and 84 (SD 17) cpm/mg DNA for cells incubated with thymidine. The substantial decreases both in the quantity of dU and the radioactivity in the dU pool seen after treatment with thymidine were statistically significant with P values of <.001 and <.025, respectively (paired t-test).

DISCUSSION

The average ratio of (A + T)/(G + C) in the DNA of normoblastic marrow samples was 1.40, which is similar to the
Table 2. Micromoles of Deoxynucleoside and Radioactivity in Each Deoxynucleoside Pool, Expressed per Milligram of Hydrolyzed DNA Applied to the Aminex A6 Column

<table>
<thead>
<tr>
<th>Deoxynucleoside</th>
<th>μmol/mg DNA</th>
<th>Radioactivity (cpm/mg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dUSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dU</td>
<td>18</td>
<td>0.036</td>
</tr>
<tr>
<td>dT</td>
<td>18</td>
<td>0.963</td>
</tr>
<tr>
<td>dC</td>
<td>18</td>
<td>0.649</td>
</tr>
<tr>
<td>dA</td>
<td>18</td>
<td>0.739</td>
</tr>
<tr>
<td>dG</td>
<td>18</td>
<td>0.568</td>
</tr>
<tr>
<td>Normal dU</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>High dU</td>
<td>10</td>
<td>388t</td>
</tr>
</tbody>
</table>

Significance of difference from corresponding value for marrow aspirates showing normal dUSV suppression test values (dUSV) calculated using the t-test: *P < .01; tP < .001, SP < .05, §not significant.

value of 1.45 obtained by earlier investigators. In the present study, DNA hydrolysates from human marrow samples showing normal dUSVs contained either no detectable dU or small amounts of dU. Furthermore, after incubation with 3H-uridine 13 of 14 of such hydrolysates showed a little radioactivity in the dU fraction obtained from DNA. Thus, there seems to be a minor degree of uracil misincorporation into DNA in normoblastic bone marrows.

Marrow cells from patients with a high dUSV contained much larger amounts of dU in their hydrolyzed DNA and, after incubation with 3H-uridine 13 of 14 of such hydrolysates showed a little radioactivity in the dU fraction obtained from DNA. Thus, there seems to be a minor degree of uracil misincorporation into DNA in normoblastic bone marrows.

There is no information on the activity of dUTPase in Bl2- or folate-deficient marrow cells, and one study has shown normal uracil N-glycosidase activity in Bl2-deficient cells. There is evidence that misincorporation of uracil is normally prevented or limited via the action of dUTPase that breaks down any dUTP formed and of uracil N-glycosidase that excises any uracil misincorporated into DNA, leaving a strand break (apyrimidinic site) that is rapidly repaired.

These mechanisms seem to be overwhelmed when a substantial amount of dUTP is formed intracellularly. There is no information on the activity of dUTPase in Bl2- or folate-deficient marrow cells, and one study has shown normal uracil N-glycosidase activity in Bl2-deficient cells. Despite the absence of data on dUTPase activity, it seems likely that the marked uracil misincorporation observed in megaloblastic marrows is related not to reduced activity of this enzyme but to a pile-up of dUMP and, consequently, dUTP caused by a failure of methylation of dUMP in the absence of adequate concentrations of dTTP at the DNA replication fork, uracil may become incorporated from the expanded intracellular pool of dUTP into DNA. This view is supported by the results of experiments into the effects on uracil misincorporation of incubating (1) normoblastic marrow cells with the dihydrofolate reductase inhibitor, MTX, for 16 hours, and (2) vitamin Bl2- and/or folate-deficient cells with thymidine for 16 hours and subsequently incubating both types of cell with 3H-uridine. The treatment with MTX would be expected to impair the 5,10-methylentetrahydrofolate-dependent methylation of dUMP to...
The treatment with thymidine to increase the intracellular pool of dTTP. MTX caused a statistically significant increase in the incorporation of radioactivity into the dU pool obtained from hydrolyzed DNA (ie, caused in increased uracil misincorporation) and thymidine caused statistically significant reductions in both the quantity of dU per milligram DNA and the radioactivity in the dU pool per milligram DNA (ie, caused a reduction in uracil misincorporation).

In certain circumstances, the misincorporated uracil in the newly synthesized daughter DNA strands of megaloblasts may well be subjected to excision at a greater rate than repair, leading to many strand breaks and, eventually, to DNA degradation. Experimentally induced misincorporation of uracil into the DNA of human lymphocytes has been shown to lead to DNA degradation in this way.28 A curious finding in our study was that there were more micromoles of dU + dT per milligram of DNA in megaloblastic marrows than in normoblastic marrows (1.199 v 0.999, respectively). This suggests that uracil misincorporation leads in some way to the formation of multiple single-stranded copies of normally thymine-rich segments of DNA. Such a possibility is supported by the observation that the maximum Feulgen absorbance (apparent maximum DNA content) achieved by individual cells is greater in PHA-stimulated normal human lymphocytes grown in folate-deficient medium than in those grown in folate-supplemented medium.29

It has been shown that some vitamin B12- and folate-deficient megaloblasts suffer from a variety of disturbances. These include a gross abnormality of erythroblast proliferation,30-32 impairment of protein biosynthesis,33 non-specific ultrastructural abnormalities,34 and the presence of increased amounts of immunoglobulin on the cell surface.35 Such abnormalities, which were most marked in the more mature erythroblasts, are clearly related to the gross ineffectiveness of erythropoiesis in vitamin B12 and folate deficiency. Considerable uracil misincorporation into DNA followed by nuclear dysfunction and DNA degradation may well be one of the important disturbances underlying the above abnormalities.

The explanation for the failure of some previous investigators to show uracil misincorporation in megaloblastic marrow is uncertain but may be related to differences in the method used to separate the deoxynucleosides. Previous workers have used paper chromatography, a technique in which trailing has been found to cause problems.15 The present study used Aminex A6 column chromatography, which permitted the separation of larger volumes than by paper chromatography and provided reliable separation of dU from dT with no trailing.

In conclusion, our data indicate that a fundamental abnormality in B12- or folate-deficient megaloblasts is in the quality of DNA formed, the DNA containing substantial amounts of misincorporated uracil. However, because there was no clear correlation between the degree of anemia and either the amount of dU in DNA or the radioactivity in the dU fraction, other intracellular factors seem to play a role in determining the extent of ineffectiveness of hematopoiesis in a particular B12- or folate-deficient patient. One such factor may be the availability of dTTP for repair of the apyrimidic sites resulting from uracil excision. Further work is required to determine whether uracil misincorporation into the DNA of normoblast cells is specific for megaloblastic hematopoiesis caused by impaired methylation of dUMP.

REFERENCES
URACIL IN DNA OF MEGALOBLASTS

Bone marrow cells from vitamin B12- and folate-deficient patients misincorporate uracil into DNA

SN Wickramasinghe and S Fida