Recent studies using anion exclusion chromatography have suggested that uracil is misincorporated into the DNA of patients with megaloblastic anemia to levels detectable by nonradioactive methods. We have investigated the nucleotide composition of DNA from the bone marrow mononuclear cells of eight patients with cobalamin deficiency and compared this with that found in normal subjects. The median level of uracil in the megaloblastic group was 0.089 mol% of cytosine (approx. 0.02 mol% of all bases in DNA), which was similar to that found in the control group (median 0.085 mol% of cytosine) and may be attributable, at least in part, to artefactual deamination of deoxycytidine monophosphate during the DNA hydrolysis. Our findings give no support for the view that, by overwhelming the uracil N-glycosidase mechanism, the degree of uracil misincorporation in megaloblastic anemia is sufficient to increase the steady state level of uracil in the DNA by amounts detectable by nonradioactive methods. Using high performance liquid chromatography, we have also demonstrated normal levels of methylcytosine in the DNA of megaloblastic subjects.

**Materials and Methods**

**Patients.** The diagnosis of cobalamin deficiency was made on the basis of anemia, macrocytosis, low-serum cobalamin (Simul-TRAC radioassay, Becton Dickinson, Oxford, UK), megaloblastic features in the bone marrow, and response to parenteral cobalamin therapy (see Table 1).

**Controls.** Bone marrow samples were obtained from four healthy subjects at hip replacement surgery and from three patients undergoing staging bone marrow biopsies for lymphoma (bone marrows not involved with disease). These studies were approved by the local ethics committee.

**Techniques.** All bone marrow was taken into heparinized phosphate buffered saline and processed immediately. The mononuclear cells were separated by density gradient centrifugation using lymphocyte separation medium (GIBCO BRL, Paisley, UK) and frozen at −70°C until DNA extraction.

**DNA extraction.** A modification of the method of Zamenhof was used. The cells were thawed and suspended in 1 mL 0.1M EDTA (pH 7.35). To this, sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% and proteinase K (Sigma, Poole, Dorset, UK) to a final concentration of 100 μg/mL. The solution was incubated at 56°C for 2 hours, and the nucleic acid extracted with NaCl (final concentration 2 mol/L). After cooling to 4°C and centrifugation for 45 minutes, the supernatant was precipitated in 100% ethanol and washed in 70% ethanol. The nucleic acid extract was air dried and dissolved in a solution of 0.14 mol/L NaCl/0.015 mol/L sodium citrate.

**Removal of RNA from DNA preparation.** Nucleic acid was incubated with Ribonuclease A (100 μg/mL) and Ribonuclease T1 (2,000 U/mL) for 2 hours at 37°C, extracted with phenol, and precipitated in 100% ethanol.

**Enzymatic hydrolysis of purified DNA.** DNA was dissolved in a deoxyribonuclease I (DNase I) digestion buffer (10 mmol/L Tris-HCl pH 7.2, 0.1 mmol/L EDTA, 4 mmol/L Magnesium chloride) and hydrolyzed by addition of DNase I (50 μg/mL, GIBCO BRL) at 37°C for 14 hours, followed by addition of two volumes of 30 mmol/L sodium acetate (pH 5.2). To this, zinc sulphate and nuclease P1 were added, each to a final concentration of 50 μg/mL and incubated for a further 7 hours. A total of 50 to 200 μL of the reaction mixture (containing deoxyribonucleotide 5'-monophosphates) was then analyzed by HPLC, while the remainder was further hydrolyzed to deoxyribonucleosides by the addition of bacterial phosphatase.

**From the Department of Haematology, University of Wales College of Medicine, Heath Park, Cardiff, Wales, UK.**

**Submitted May 26, 1995; accepted October 16, 1995.**

**Supported by the Medical Research Council of the United Kingdom.**

**Address reprint requests to B.H. Ramsahoye, MD, Department of Haematology, University Hospital of Wales, College of Medicine, Heath Park, Cardiff, UK.**

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.
(Escherichia coli) alkaline phosphatase (BAP, 2 U/mL, 37°C, 7 hours. Sigma). These samples were then analyzed immediately by HPLC or frozen at −70°C until analysis.

HPLC analysis. The HPLC equipment comprised a Waters pump, Waters U6K injector, Waters 440 U.V. absorbance detector, Technicon dual channel chart recorder, Jones 25 cm pump, Waters U6K injector, Waters 440 U.V. absorbance detector, HPLC column (5 pm Apex ODS). The mobile phase (1 mL/min) was made by dissolving 50 mmol (NII)HPO₄ in 1 L of 50 mmol/L orthophosphoric acid and the pH adjusted to 4.1 with 1 mol/L orthophosphoric acid.

Tests of reproducibility. HL60 cells harvested in the exponential phase of growth in RPMI 1640 medium (10% fetal calf serum) were divided into five equal aliquots. DNA extractions and hydrolyses were performed independently to obtain the coefficient of variation for the ratios deoxyuridine to deoxycytidine (dU/dC) and methyl-deoxycytidine monophosphate to total deoxycytidine monophosphates (mdCMP/mdCMP + dCMP).

Tests of artefactual deamination of deoxycytidine. (1) Spontaneous deamination of dCMP: A standard solution of dCMP in DNase I (21 hours) and nuclease P1 (7 hours). In the case of dCMP, the sample was then treated with BAP as above to allow detection of dU by HPLC.

The effect of using a crude DNase I preparation (Sigma, catalogue no. DN 25) on the level of measured uracil in calf thymus DNA (Sigma), was also assessed. This was to demonstrate the extent to which impurities in the DNase I might artefactually produce deamination of deoxycytidine.

Identification of peaks. Deoxyribonucleotide, ribonucleotide, deoxyribonucleoside, and ribonucleoside standards (Sigma) were run to determine their retention times. The presence of dU was confirmed by spiking a sample with a standard preparation of dU. Quantitation of deoxyribonucleotide-5'-monophosphates was achieved by calibrating the system for peak heights using seven duplicate dilutions of a mixture of nucleotide standards. Similarly, dC and dU were calibrated using dilutions of standard dC and mixtures of dC and dU. The compounds were quantitated according to Beer’s law. Regression lines were drawn using Minibat software (Minibat Inc, State College, PA; least squares approach). The relationship between peak height and quantity of nucleotide/nucleoside injected onto the column was linear within the range of concentrations used in the study. The regression equations for the calibrations and values for $R^2$ are given in Table 2.

Table 1. Presentation and Follow-up Data for Cobalamin Deficient Patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>Hb (g/dL)</th>
<th>Serum Cobalamin (ng/L)</th>
<th>Serum Folate (µg/L)</th>
<th>MCV (FL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45/M</td>
<td>PA(IFA+)*</td>
<td>6.4</td>
<td>&lt;50</td>
<td>3.9</td>
<td>135</td>
</tr>
<tr>
<td>2</td>
<td>69/M</td>
<td>PA(IFA+)*</td>
<td>13</td>
<td>&lt;50</td>
<td>8.8</td>
<td>110.7</td>
</tr>
<tr>
<td>3</td>
<td>55/F</td>
<td>PA(IFA+)*</td>
<td>10</td>
<td>51</td>
<td>5.7</td>
<td>115</td>
</tr>
<tr>
<td>4</td>
<td>49/F</td>
<td>PA(IFA+)*</td>
<td>11.2</td>
<td>&lt;50</td>
<td>&gt;20</td>
<td>107.1</td>
</tr>
<tr>
<td>5</td>
<td>70/F</td>
<td>PA(IFA+)*</td>
<td>6.7</td>
<td>71</td>
<td>8.3</td>
<td>118.7</td>
</tr>
<tr>
<td>6</td>
<td>60/F</td>
<td>PA*</td>
<td>10.8</td>
<td>&lt;50</td>
<td>8.3</td>
<td>139.3</td>
</tr>
<tr>
<td>7</td>
<td>81/M</td>
<td>PA+</td>
<td>12.0</td>
<td>51</td>
<td>13.1</td>
<td>109</td>
</tr>
<tr>
<td>8</td>
<td>45/M</td>
<td>Crohns,iilectomy†</td>
<td>12.4</td>
<td>&lt;50</td>
<td>&gt;20</td>
<td>115</td>
</tr>
</tbody>
</table>

Abbreviations: PA, pernicious anemia; IFA, intrinsic factor antibody; */**/*** , mild/moderate/marked megaloblastosis; MCV, mean cell volume.

* Marked megaloblastosis.
† Mild megaloblastosis.
‡ Moderate megaloblastosis.

Table 2. Summary of Calibration System Used for the Quantitation of Nucleotides and Nucleosides

<table>
<thead>
<tr>
<th>Nucleotide/Nucleoside</th>
<th>$\lambda_{max}$ (nm, pH 7.4)</th>
<th>Detection $\lambda$ (nm)</th>
<th>Chart Recorder Range (mV)</th>
<th>Regression Equation ($X$ nanomoles, $Y$ inches)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>272.7</td>
<td>280</td>
<td>10</td>
<td>$Y = 0.16 + 0.51X$</td>
<td>0.998</td>
</tr>
<tr>
<td>mdCMP</td>
<td>278</td>
<td>280</td>
<td>0.4</td>
<td>$Y = 0.47 + 19.1X$</td>
<td>0.998</td>
</tr>
<tr>
<td>TMP</td>
<td>267.3</td>
<td>280</td>
<td>10</td>
<td>$Y = 0.14 + 0.22X$</td>
<td>0.996</td>
</tr>
<tr>
<td>dGMP</td>
<td>260.1</td>
<td>280</td>
<td>10</td>
<td>$Y = 0.12 + 0.21X$</td>
<td>0.993</td>
</tr>
<tr>
<td>dAMP</td>
<td>256.6</td>
<td>280</td>
<td>10</td>
<td>$Y = 0.05 + 6.8 \times 10^2X$</td>
<td>0.954</td>
</tr>
<tr>
<td>dC</td>
<td>270</td>
<td>254</td>
<td>10</td>
<td>$Y = 0.05 + 0.12X$</td>
<td>0.990</td>
</tr>
<tr>
<td>dU</td>
<td>263</td>
<td>254</td>
<td>0.4</td>
<td>$Y = -0.004 + 11.5X$</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Abbreviations: $s$, estimate of $\sigma$, the estimated standard deviation about the regression line (measured in inches when nucleotide quantity is taken as the predictor and in nanomoles when peak height is taken as the predictor); $\lambda$, wavelength of absorption; $\epsilon$, extinction coefficient.
composition of DNA in cobalamin deficiency

controls dCMP mdCMP dGMP TMP dAMP mdCMP
uracil deamination of the dC or dCMP standards. The level of SD 0 hours, 0.006% at 0.005% at 0.017% and the chromatographically purified product (0.024%).

discussion

Current understanding of methionine metabolism suggests two ways in which cobalamin deficiency might affect DNA methylation. Firstly, methylcobalamin is a cofactor for methionine synthase, the enzyme that regenerates methionine from homocysteine. Failure to regenerate methionine could lead to a reduction in S-adenosylmethionine, the methyl donor for DNA cytosine methyltransferase. Despite this, the empirical evidence both in this study and in the only other previous study addressing this issue, demonstrates that genome-wide methylation is unaffected in cobalamin deficiency. Also studies addressing the methylation status of hypomethylation. It is likely, therefore, that dietary methionine is sufficient for S-adenosylmethionine generation in cobalamin deficiency. Secondly, one study has also demonstrated that in vitro, low cobalamin concentrations (0.3 μmol/L) enhance the de novo DNA cytosine methyltransferase reaction in the presence of 10 μmol/L S-adenosylmethionine, whereas high concentrations are inhibitory. Hence, when cellular cobalamin levels are low, overall DNA methylation could be maintained because of enhanced methylcobalamin cofactor activity.

The possibility of uracil misincorporation in megaloblastic DNA was first suggested by Luzzatto et al after experiments based on measuring [3H]uridine incorporation into the DNA of megaloblastic bone marrow cells in culture. These findings were not confirmed at the time in patient material, though studies on cell lines treated with antifolates suggested

that misincorporation could be induced.16,17 Goulian et al16 detected no uracil in untreated human lymphoid cells (<1 in 10^12 bases) and 1 labelled uracil in 10^6 bases (0.0001 mol%) in the methotrexate treated cells after a 2-hour incubation with [5-^3H]dU (a 10^9-fold increase). The extent to which this incorporation of labelled uracil reflects the actual content of total DNA uracil is difficult to estimate, and presumably depends on the proportion of labelled to nonlabelled dUTP in the soluble nucleotide pool. Recently studies employing anion exclusion HPLC have suggested misincorporation of much larger amounts of uridine (12 mol%) in both megaloblastic bone marrow and HL60 cells grown in folate and cobalamin deficient medium. These findings were supported by the results of [5-^3H]uridine incorporation studies.17

The level of uracil found in the present study is approximately 100 to 1,000 times lower than the mean level found using anion exclusion HPLC,6 and no significant difference was found between megaloblastic and control groups (Table 3). We think it likely that the dU detected in our study arises, at least in part, as a result of spontaneous deamination of dCMP at 37°C. Failure to detect a difference in uracil misincorporation between patient and control groups in our study could be due to the masking of differences by artefactual deamination of cytosine to uracil. Incubation of a standard dCMP preparation at 37°C produced a time dependent increase in dUMP, 0.015% of dCMP being converted to dUMP in 24 hours. It is also possible that additional deamination might have occurred during cleavage of the phosphodiester bonds in DNA, but this is difficult to test. The amount of dU/dC detected in patient and control samples, though very low (median 0.08% of cytosine), varied over a wide range in both groups. This was despite low values of s (mean square error) for each of the respective standards calibrated (see Table 2). Where material was available, replicate samples also showed some variation in dU/dC (Table 4). This was out of proportion with the variation expected from the
COMPOSITION OF DNA IN COBALAMIN DEFICIENCY

coefficient of variation (15%), but far less than the wider variation seen within both groups (see Table 3). In contrast, the mol% values for methylcytosine and the major bases showed little variation and were in accordance with the results of others.10,18

With reference to the very low level of uracil detected in this study compared with that reported by others,6 we think it unlikely that our technique underestimates the true level present. Failure of the hydrolytic enzymes (DNase I and nuclease P1) to cleave dUMP from DNA had it been present, would have produced a marked discrepancy in base ratios. In addition, long incubation times were used to ensure complete hydrolysis. Also, of the eight patients presented here, at least four had markedly megaloblastic marrows suggesting no correlation between the degree of megaloblastosis and the presence of uracil in DNA.

However, two important differences in methodology should be considered. First, it is possible that the isolation of marrow mononuclear cells by density gradient centrifugation (as used in this study) may have excluded any apoptotic or otherwise damaged uracil containing cells from the analysis. Though this possibility warrants further investigation, the amount of apoptosis detected morphologically in even the grossly megaloblastic bone marrow smears was negligible, and erythroid megaloblasts are not removed by density gradient centrifugation (personal observations). Second, the studies, which have shown a high level of uracil misincorporation in megaloblastic bone marrows, have used bone marrow culture in the presence of [5-3H]uridine. It could be that bone marrow culture results in more apoptotic uracil containing cells than is the case in vivo, either because of some artefact related to the culture conditions or because of failure of macrophages to phagocytose any apoptotic cells generated in vitro. Indeed, active phagocytosis of damaged cells in vivo could account for the general absence of apoptotic cells in megaloblastic bone marrow smears despite evidence of ineffective hemopoiesis and cell death by ferrokinetic studies. Such a scenario has been proposed to explain the absence of morphological evidence of apoptosis in myelodysplasia, despite the evidence for ineffective hemopoiesis.19

It seems likely, however, that the marked degree of apparent uracil incorporation reported by others6 is artefactual. Importantly, a concurrent reduction in thymidine was not found in the study on megaloblastic bone marrow.6 Their findings may be attributable either to artefactual cytosine deamination or to incorrect identification of chromatogram peaks, as might be the case if RNA contaminated the DNA preparation. Indeed, we have shown (data not presented) that RNA hydrolysis with ribonuclease A alone, as used in the aforementioned study, is not sufficient to hydrolyze all the contaminating RNA, even if the incubation is allowed to proceed for 6 hours. This is presumably due to the nucleotide preference of ribonuclease A, which cleaves RNA to leave 3' phosphohistidinol nucleotides ending in Cp and Up.20 Ribonuclease T1 cleaves RNA to leave 3' phosphohistidinol nucleotides ending in Gp. The combination of ribonuclease A and ribonuclease T1, as used in this study, was effective in removing all but a trace of the RNA (Fig 2).

Despite the absence of supportive evidence in this study, the uracil misincorporation hypothesis would explain the karyotypic abnormalities and ineffective hemopoiesis seen in megaloblastic anemia. It could be that excision of misincorporated uracil is rapid, as demonstrated by Goulian et al,18 and that it is, therefore, only found in trace quantity, if at all. The more crucial abnormality in megaloblastic anemia may be repair of the apyrimidinic sites by DNA polymerase after excision of uracil by uracil N-glycosidase. This is dependent on adequate supplies of thymidine triphosphate (TTP). Failure to convert dUMP to TTP in megaloblastic anemia should hypothetically lead to reduced cellular TTP concentrations, but the evidence for this has been conflicting.16,21

ACKNOWLEDGMENT

We thank Dr P. Bentley for providing bone marrow samples and Dr D. Bowen and Dr D. Culligan for advice in preparation of this manuscript.

REFERENCES


2. Dormer P: Kinetics of erythropoietic cell proliferation in nor-
mal and anaemic man. A new approach using quantitative \(^{14}\)C autoradiography. Prog Histochem Cytochem 6:1, 1973


6. Wickramasinghe SN, Fida S: Bone marrow cells from vitamin \(B_{12}\) and folate-deficient patients misincorporate uracil into DNA. Blood 83:1656, 1994


Nucleic acid composition of bone marrow mononuclear cells in cobalamin deficiency

BH Ramsahoye, AK Burnett and C Taylor