Bone Marrow Cells From Vitamin B_{12}- and Folate-Deficient Patients Misincorporate Uracil Into DNA

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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 B_{12} or folate deficiency were examined for misincorporation of uracil into DNA. Cells were incubated with [5-^{3}H] uridine for 2 hours and their DNA extracted. The DNA was hydrolyzed to deoxyribonucleosides with DNase 1, phosphodiesterase 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 B_{12}- 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 B_{12} and folate deficiency.

IT IS OFTEN CONSIDERED that the megaloblastic hematopoiesis found in vitamin B_{12} or folate deficiency is caused by impaired methylation of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (dTMP),[1-4] which in turn leads to a reduced supply of thymidine triphosphate (dTTP) at the DNA replication fork and a reduced rate of DNA strand replication.[5-7] However, the literature on rates of DNA chain elongation in these deficiency states is contradictory, with impaired rates reported in phytohaemagglutinin-stimulated blood lymphocytes derived from B_{12}- or folate-deficient patients[6,7] and normal rates reported in B_{12}-deficient bone marrow cells.[8] Furthermore, although it would be expected that the duration of the DNA synthesis (S) phase of megaloblasts would be markedly increased if there was a substantial slowing of DNA chain elongation, some data indicate that the S phase of early polychromatic megaloblasts is either normal or only modestly increased.[9,10] In addition, HL60 cells cultured for 12 to 34 days in a medium containing 100 nmol/L folic acid showed reduced thymidine synthetase activity, gave an abnormal dU-suppressed value, and became slightly macrocytic but had a normal doubling time and a near-normal S phase.[11] These discrepancies suggest that an impaired rate of DNA strand elongation may not be the only or even the most important abnormality of DNA replication in B_{12} or folate deficiency. An alternative possibility is that the composition of the DNA synthesized in these deficiency states may be abnormal as a result of the misincorporation into DNA of uracil in lieu of thymine. Such misincorporation may result from the impairment of the methylation of dUMP and a consequent increase in the concentration of dUTP at the DNA replication fork. However, the reports by Luzzatto et al[12-14] that uracil is misincorporated into the DNA of vitamin B_{12}- or folate-deficient megaloblastic marrow cells were not confirmed[15] and have therefore been largely ignored. Nevertheless, there is good evidence for uracil misincorporation in two human lymphoma cell lines treated with inhibitors of dihydrofolate reductase.[16,17] Furthermore, we recently found that uracil became misincorporated into the DNA of HL60 cells deprived in vitro of folate, vitamin B_{12}, or both.[18] In this article we report the results of a reexamination of the possibility that uracil misincorporation occurs in folate- or vitamin B_{12}-deficient bone marrow cells.

MATERIALS AND 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 5×10^6/mL. An aliquot of the cell suspension was used for the performance of a dU suppression test using a microtiter plate method.[19] Another aliquot was incubated with 0.5 μCi/mL [5-^{3}H] 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) containing 10% (vol/vol) human group AB serum. The cells were then incubated for 16 hours at 37°C both with and without 10 μmol/L methotrexate (MTX; 4-amino-10-methylfblic acid) or 10 μmol/L thymidine (both from Sigma Chemical Company, Poole, Dorset, UK), washed with TBH and resuspended in TBH before labeling with [5-^{3}H] uridine and isolating the 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 2½ vol of cold ethanol (−20°C), spooled and cleaned, as described by Saffhill et al.[20] The cleaned DNA was dissolved in water overnight and the total yield calculated

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Submitted June 15, 1993; accepted October 28, 1993.

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0006-4971/94/8306-0118$3.00/0

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 Tris/HCl, pH 7.4, 5 mmol/L $MgCl_2$, 3 mmol/L sodium azide, and 1 μmol/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-performance 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 10 mmol/L ammonium bicarbonate buffer (pH 8.0). The DNA hydrolysate was loaded onto the Aminex A6 column 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 10^4, dT 9.6×10^3, deoxycytidine (dC) 9×10^3, deoxyadenosine (dA) 1.5×10^4, and deoxyguanosine (dG) 1.37×10^4; 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 hydrolysates 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,[18] the conversion of ^{3}H-uridine into ^{3}H-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-^{3}H] uridine used in this study. Following conversion to ^{3}H-dUMP as above, the ^{3}H-dUMP would be methylated to ^{3}H-dTMP and eventually converted to ^{3}H-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

Case	Sex	Age (yrs)	Hb (g/dL)	MCV (FL)	Serum B_{12} (ng/L)	Serum Folate (μg/L)	Red Blood Cell Folate (μg/L)	dUSV (%)	Diagnosis
PHa	M	73	6.2	115	56		445	51.0	Pernicious anemia
PHe	M	78	6.3	131	173	1.1	69	29.8	Dietary folate deficiency, bronchopneumonia
MM	F	60	3.2	151	253		0	40.9	Dietary folate deficiency
MG	F	86	5.9	141	11	6.9	73	40.0	Pernicious anemia
FB	F	88	7.5	113	37	1.4	76	49.5	Dietary B_{12} and folate deficiency
MS	F	39	6.7	125	41	2.7	267	34.1	Pernicious anemia
LQ	F	79	7.8	120	23	3.5	142	48.3	Pernicious anemia
HF	F	61	5.9	117	164	1.0	40	32.9	Dietary B_{12} and folate deficiency
EO	F	65	11.6	105	86	3.8	112	28.9	Pernicious anemia
JB	F	70	8.9	130	68	1.9	296	57.7	Pernicious anemia
MGI	F	75	11.5	117	81	2.6	188	49.9	Pernicious anemia

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

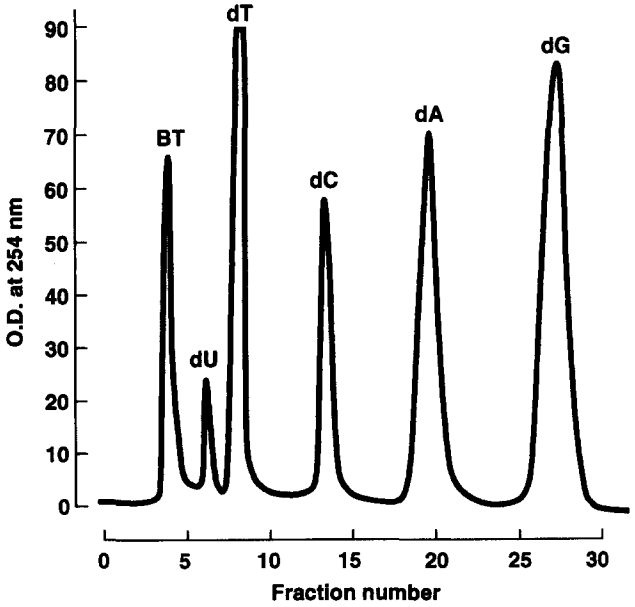

Fig 1. Elution profile of deoxyribonucleosides obtained by Aminex A6 chromatography of a DNA hydrolysate prepared from a marrow sample showing a normal dUSV. BT, breakthrough peak; dU, deoxyuridine; dT, deoxythymidine; dC, deoxycytidine; dA, deoxyadenosine; dG, deoxyguanosine. Note the small dU peak.

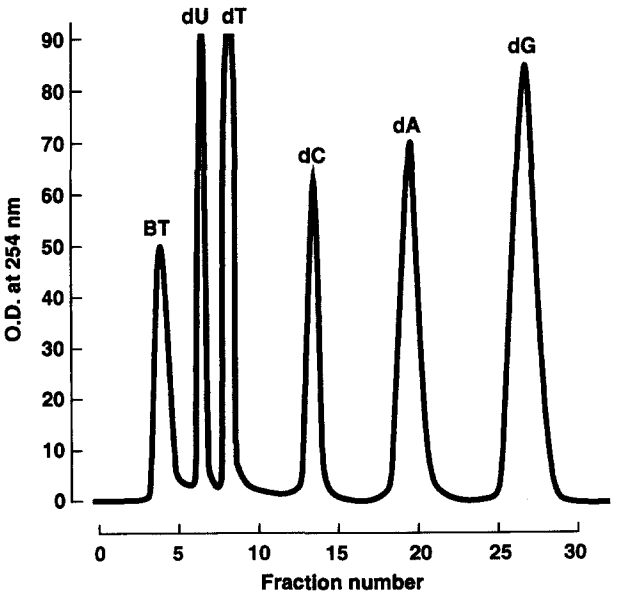

Fig 2. Elution profile of deoxyribonucleosides obtained by Aminex A6 chromatography of a DNA hydrolysate prepared from a marrow sample of a patient with megaloblastic anemia caused by vitamin B_{12} deficiency. BT, breakthrough peak; dU, deoxyuridine; dT, deoxythymidine; dC, deoxycytidine; dA, deoxyadenosine; dG, deoxyguanosine. Note that the dU peak is considerably larger than in Fig 1.

Table 2. Micromoles of Deoxynucleoside and Radioactivity in Each Deoxynucleoside Pool, Expressed per Milligram of Hydrolyzed DNA Applied to the Aminex A6 Column

			μmol/mg DNA		Radioactivity (cpm/mg DNA)		
dUSV	Deoxy-nucleoside	No.	Mean	SD	No.	Geometric Mean	95% Reference Limits
Normal	dU	18	0.036	0.041	14	22	3-159
	dT	18	0.963	0.144	14	276	64-1185
	dC	18	0.649	0.143	14	61	14-260
	dA	18	0.739	0.173	14	40	2-574
	dG	18	0.568	0.084	14	34	2-464
High	dU	11	0.378*	0.120	10	388†	261-576
	dT	11	0.821‡	0.165	10	262§	51-1356
	dC	11	0.586§	0.104	10	106§	7-1701
	dA	11	0.674§	0.163	10	35§	5-241
	dG	11	0.560§	0.075	10	28§	4-187

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

value of 1.45 obtained by earlier investigators.[22] 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 ^{3}H-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 ^{3}H-uridine, contained 19 times more radioactivity in the dU fraction than marrow cells with a normal dUSV. Because the patients with a high dUSV had megaloblastic hematopoiesis caused by B_{12} and/or folate deficiency, these findings indicate that B_{12}- or folate-deficient marrow cells suffer from a considerable degree of misincorporation of uracil into DNA. On average, the proportion of thymine replaced by uracil seemed to be about 30% (after correction for artefactual production of dU by deamination of dC), an amount similar to that reported in the DNA of T4 bacteriophages growing in host cells deficient in both dUTPase and uracil DNA glycosidase activity.[23] There is evidence that misincorporation of uracil is normally prevented or limited via the action of dUTPase that breaks down any dUTP formed[24] and of uracil N-glycosidase that excises any uracil misincorporated into DNA, leaving a strand break (apyrimidinic site) that is rapidly repaired.[25,26] These mechanisms seem to be overwhelmed when a substantial amount of dUTP is formed intracellularly.[16,17] There is no information on the activity of dUTPase in B_{12}- or folate-deficient marrow cells, and one study has shown normal uracil N-glycosidase activity in B_{12}-deficient cells.[27] 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 B_{12}- and/or folate-deficient cells with thymidine for 16 hours and subsequently incubating both types of cell with ^{3}H-uridine. The treatment with MTX would be expected to impair the 5,10-methylenetetrahydrofolate–dependent methylation of dUMP to

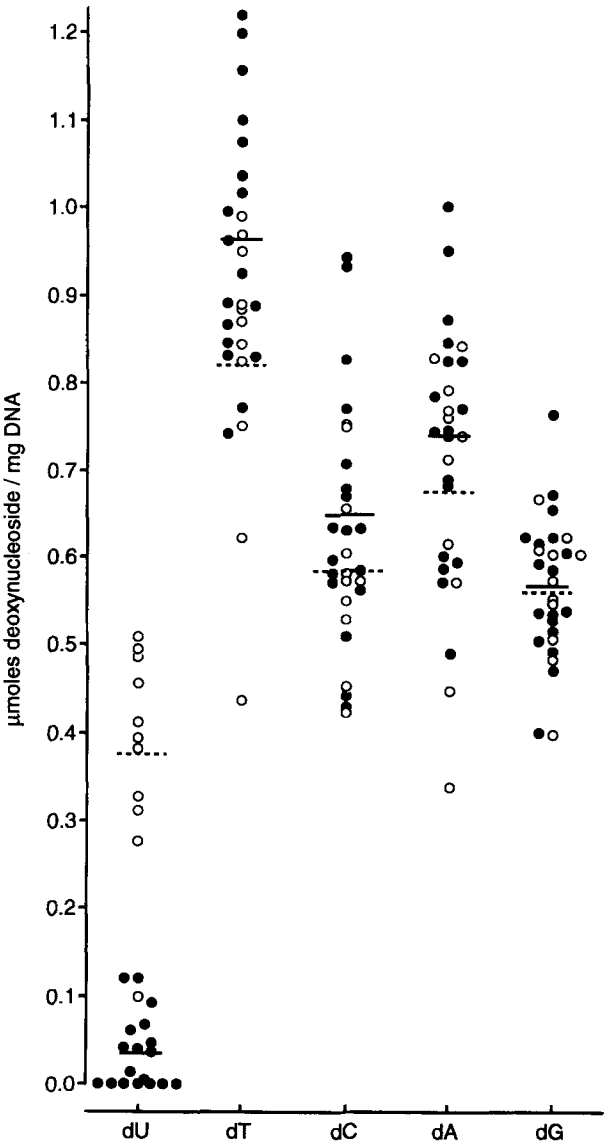

Fig 3. Individual values for the quantity of deoxynucleoside in each of the five deoxynucleoside peaks obtained by Aminex A6 chromatography of hydrolyzed DNA from 29 bone marrow aspirates. The continuous horizontal lines represent the arithmetic means for marrow samples showing normal dUSVs (●) and the interrupted horizontal lines, the arithmetic means for samples showing high dUSVs (○).

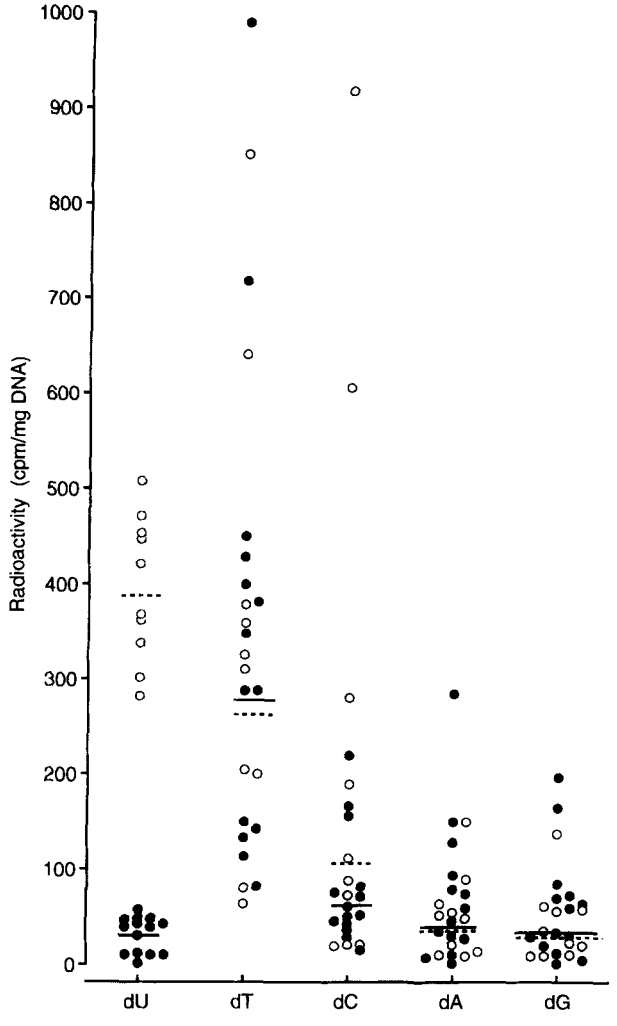

Fig 4. Individual values for the radioactivity in each of the five deoxynucleoside pools obtained by Aminex A6 chromatography of hydrolyzed DNA from 24 bone marrow aspirates. The continuous horizontal lines represent the geometric means for marrow samples showing normal dUSVs (●) and the interrupted horizontal lines, the geometric means for samples showing high dUSVs (○).

dTMP and 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 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 B_{12}- and folate-deficient megaloblasts suffer from a variety of disturbances. These include a gross abnormality of erythroblast proliferation,[30-32] impairment of protein biosynthesis,[32] 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 B_{12} 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 B_{12}- 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 B_{12}- or folate-deficient patient. One such factor may be the availability of dTTP for repair of the apyrimidinic sites resulting from uracil excision. Further work is required to determine whether uracil misincorporation into the DNA of marrow cells is specific for megaloblastic hematopoiesis caused by impaired methylation of dUMP.

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1994 83: 1656-1661

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.