The effect of folate analogues on thymidine utilization by human and rat marrow cells and the effect on the deoxyuridine suppression test

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Summary

The deoxyuridine (dU) suppression test assesses the capacity of marrow cells or activated lymphocytes to convert exogenous dU into thymidine. In addition, the effect of the added cobalamin or folate on the result is used to determine the nature of the deficiency in megaloblastic marrows. This study shows that folates have a considerable effect on the uptake of labelled thymidine by marrows from patients with megaloblastic anaemia in the absence of added dU. Less thymidine was taken up by megaloblastic marrow cells if they were first incubated with 5-formyltetrahydrofolate, 10-formyltetrahydrofolate or tetrahydrofolate. 5-Methyltetrahydrofolate increased thymidine uptake in cobalamin-deficient marrows and reduced thymidine uptake in folate-deficient marrows. These results could be explained if added folates facilitated utilization of endogenous dU. However, the addition of folates or cobalamin did not affect the validity of a dU suppression test with human marrow. More marked changes were present on the addition of folates to rat marrow cells both from control and nitrous oxide-treated animals.

Introduction

Thymidine synthesis is impaired in cells from patients with untreated megaloblastic anaemia. At a biochemical level there is depressed conversion of deoxyuridine (dU) to deoxythymidine. Both folate and cobalamin are required for this step which involves the transfer of a single-carbon unit ($-\text{CH}_2$ or methylene) from methylenetetrahydrofolate to dU. During this transfer, by thymidylate synthetase, the methylene group is reduced to methyl ($-\text{CH}_3$). The dU suppression test performed on marrow samples tests this step by noting first, how the cells use dU to make thymidine and, subsequently, if their thymidine requirements have not been met from dU, how much preformed labelled thymidine they are able to use (Killman, 1964; Metz *et al.*, 1968). If the marrow cells are able to convert dU into thymidine effectively very little of the exogenous

thymidine will be used. Cells that do not use dU normally will take up larger amounts of added labelled thymidine. Experience indicates that the test is abnormal in almost all patients with megaloblastic anaemia (Chanarin, 1979). The defect is corrected on the addition of cobalamin to marrow from cobalamin-deficient patients and the result is improved in all megaloblastic marrows on the addition of most folate compounds.

Studies using marrow from rats, both controls and animals in which cobalamin had been inactivated by nitrous oxide, made it possible to test a much wider range of folate analogues (Deacon *et al.*, 1980). The results indicated that in the rat, addition of formylfolates were much more effective in overcoming the block produced by B_{12} inactivation than other folates and that the result could be significantly affected by the addition of folate to the control tube (lacking dU). Bain (1975) and Rogers and Lietman (1977) showed that the addition of folate to the culture medium reduced uptake of ^3H -thymidine by stimulated lymphocytes. Wickramasinghe and Saunders (1979) reported lower dU values in 2 out of 8 tests when additions of vitamins were made to the control tube. Apart from this paper this aspect has not received a great deal of attention in interpreting data from the dU suppression tests.

The purpose of this study was to explore the effect on thymidine requirements of adding folate analogues to marrow from megaloblastic patients and control subjects and, secondly, to assess the effect of such modified controls on the results of the dU suppression test. Similar studies were made with rat marrow both from control and N_2O -treated animals.

Materials and methods

Patients studied

Marrow samples were obtained from 11 patients with untreated megaloblastic anaemia, 6 due to pernicious anaemia and 5 to folate deficiency. In

addition, observations were made on 4 marrows from haematologically normal patients. These had been requested as part of the investigation of the patient.

Animals

Male, Sprague-Dawley, 80- to 120-g rats were placed in a chamber in which a mixture of N₂O (50%) and oxygen (50%) was passed, and CO₂ and humidity controlled. After 3 hr the animals were killed by exsanguination preceded by an injection of sodium pentobarbitone, long bones removed, split with a scalpel and marrow washed into 5 ml cold Hanks's balanced salt solution (BSS) (pH 7.2-7.4) containing 0.5 ml preservative-free heparin (1000 i.u./ml). Control animals were left in air.

Marrow

The marrow collected in Hanks's solution was passed through 21- and 25-gauge needles to break up clumps and washed in cold Hanks's BSS. The cells were then resuspended in a known volume of Hanks's BSS containing 10% rat or 20% human serum. A cell count was performed and volume adjusted so that there were between 1.5 × 10⁶ and 7 × 10⁶ cells/ml.

Deoxyuridine suppression test

In this test a marrow cell suspension is incubated with dU. This is normally converted into thymidine which in turn is incorporated into DNA. At a second stage [³H]thymidine is added so that any requirement not met by synthesis from dU is met by utilization of [³H]thymidine. A 100% value is obtained by incubating marrow with [³H]thymidine directly without preceding dU. The result is expressed as

$$\frac{[\text{H}]thymidine uptake after dU \times 100}{[\text{H}]thymidine uptake alone}.$$

Normal rat or human marrow cells meet more than 90% of their thymidine requirements from dU so that less than 10% of [³H]thymidine is used.

Tests and controls were set up in duplicate. One marrow suspension was added to each tube followed by 10 µl of the additive (cobalamin and/or a folate analogue) and the tubes incubated at 37°C for 15 min. Deoxyuridine (Sigma) 0.33 µmol in 10 µl saline was added to one set of tubes and incubation at 37°C continued for 15 min. [³H]thymidine (1 µCi/0.2 nmol) (Amersham) in 100 µl saline was added to all tubes and incubated for 60 min. The cells were washed twice in cold phosphate buffered saline, 2 ml cold 0.5 M perchloric acid added to the cell pellet and left in ice for 10 min. The supernatant was decanted and DNA extracted in 0.5 ml 0.5 M perchloric acid for 20 min at 80°C.

[³H]thymidine was measured in 100 µl of the clear extract by liquid scintillation, appropriate correction being made by quenching.

[³H]thymidine uptake alone

The tests consisted of duplicate tubes, one set containing marrow cells and thymidine and the other set the additive (see below), marrow cells and thymidine. Volumes of reagents, incubation and processing was as for the dU suppression test.

Solutions

The following were added in the dU suppression test and in the thymidine uptake study: hydroxocobalamin (OHCbl) 1 mg/ml, DL 5-CHO-H₄PteGlu (Lederle) 3 mg/ml, PteGlu 1.5 mg/ml, DL 5-CHO-H₄PteGlu (Sigma) 3 mg/ml in 0.1 M potassium phosphate buffer pH 7.0 to which was added 10 mM 2-mercaptoethanol, DL H₄PteGlu (Sigma) 3 mg/ml in 1% potassium ascorbate pH 6.5 stored under nitrogen, 10-CHO-H₄PteGlu 3 mg/ml prepared from 5-CHO-H₄PteGlu (Sigma) by the method of Rabinowitz (1963) in 1% potassium ascorbate pH 6.5 and kept under nitrogen.

Results

The effect of ascorbate, 2-mercaptoethanol and buffer solution on [³H]thymidine uptake by marrow

These reagents were required to maintain the

TABLE 1. Key to formulae used in this paper

Formulae	Substance
F (PteGlu)	folic acid (pteroylglutamic acid)
5-CHO-H ₄ F	5-formyltetrahydrofolate
10-CHO-H ₄ F	10-formyltetrahydrofolate
OH-Cbl	hydroxocobalamin
OH-Cbl + 5-CHO-H ₄ F	hydroxocobalamin + 5-formyltetrahydrofolate
OH-Cbl + 10-CHO-H ₄ F	hydroxocobalamin + 10-formyltetrahydrofolate
OH-Cbl + 5-CH ₃ -H ₄ F	hydroxocobalamin + 5-methyltetrahydrofolate
OH-Cbl + H ₄ F	hydroxocobalamin + tetrahydrofolate
5-CH ₃ -H ₄ F	5-methyltetrahydrofolate
H ₄ F	tetrahydrofolate

stability of folate analogues used in these studies. One per cent. ascorbate pH 6.5 had no effect in the dU suppression test using rat marrow, nor was there any effect produced by the buffer solution in which 10-CHO-H₄PteGlu was dissolved. However, the 0.1 M potassium phosphate buffer with 2-mercaptoethanol used to preserve 5-CH₃-H₄PteGlu had a significant effect on the result of the dU suppression test which fell from 10.9 and 10.5% to 8.4 and 6.9% in 2 tests respectively.

The effect of cobalamin and/or folate analogues on [³H]thymidine uptake by marrow cells

In these studies, B₁₂ and/or folate were first added to marrow suspensions followed by [³H]thymidine. B₁₂ or folate were omitted from a second set of tubes to which thymidine alone was added. These served as controls. In each study the uptake of [³H]thymidine in the control (100%) was compared with that in the presence of the additive.

There were significant reductions of thymidine uptake after the additions of tetrahydrofolate and its 10-formyl derivative to rat marrow (*P*>0.001) (Table 2). Results of individual experiments in control rats are shown in Fig. 1. Generally similar results were obtained on control and N₂O-treated rats (with the exception of 10-CHO-H₄PteGlu) and the effects were more severe when both B₁₂ and folate analogues were added together.

By contrast the addition of methylfolate enhanced the uptake of thymidine.

Data using normal human marrow cells (Table 3 and Fig. 2) show that folate analogues and hydroxocobalamin did not have any significant effect on the uptake of thymidine. Nor did B₁₂ have any significant effect on cells from patients with megaloblastic anaemia due to either B₁₂ or folate deficiency. Folate analogues, however, had a highly significant effect. Tetrahydrofolate and its 5-formyl- and

10-formyl derivatives produced a fall in the amount of thymidine taken up. Methyltetrahydrofolate produced a significant fall in thymidine uptake in folate-deficient cells and a significant rise in uptake by B₁₂-deficient cells. This difference was such that the addition of methylfolate followed by thymidine completely differentiated between the group due to B₁₂ deficiency and that due to folate deficiency.

FIG. 1. Rat marrow cells were incubated with labelled thymidine alone (control) and after the addition of folate and/or hydroxocobalamin. The result is expressed as a percentage of thymidine uptake in the presence of B₁₂/folate over thymidine uptake alone. Each point is the result of a single test performed in duplicate.

The effect of cobalamin/folate on the result of the deoxyuridine suppression test

Results of the dU suppression test using marrow from rats breathing air and breathing N₂O/oxygen (1/1) are shown in Table 4 and Fig. 3. As reported elsewhere (Deacon *et al.*, 1980), exposure to N₂O impairs the synthesis of thymidine and hence the

TABLE 2. The effect of folates and/or cobalamin on the uptake of [³H] thymidine by marrow cells from air-breathing and N₂O-treated rats
Counts [³H] thymidine + additive × 100 %
Counts with [³H] thymidine alone

Additive	Air-breathing rats			N ₂ O-breathing rats		
	No.	Mean	s.d.	No.	Mean	s.d.
OHcbl	10	103	8.6	8	96	6.8
5-CHOH ₄ F	12	101	12.4	10	100	10.7
H ₄ F	8	77*	9.4	8	88*	14.7
10-CHOH ₄ F	8	84*	11.6	8	93	16.9
5-CH ₃ H ₄ F	8	117*	12.9	8	115*	13.4
F	4	101	13.8	4	102	9.9
OHcbl + 5-CHOH ₄ F	8	101	8.8	8	105	15.8
OHcbl + H ₄ F	8	37*	13.2	8	57*	20.1
OHcbl + 10-CHOH ₄ F	8	64*	19.2	8	79*	23.3
OHcbl + 5-CH ₃ H ₄ F	8	115*	9.9	8	122*	22.7
OHcbl + F	4	106	7.4	4	98	14.7

*Significant difference from control (100%) value.

TABLE 3. The effect of folates or cobalamin on the uptake of [^3H] thymidine by marrow cells from control subjects and patients with cobalamin or folate deficiency

Counts [³ H] thymidine + additive × 100 %									
Counts with [³ H] thymidine alone									
Additive	No.	Controls		No.	B ₁₂ -deficiency		No.	Folate-deficiency	
		Mean	s.d.		Mean	s.d.		Mean	s.d.
OHCbl	4	97	14.6	6	94	8.2	5	91	10.9
5—CHO—H ₄ F	4	94	10.9	6	75*	14.8	5	80*	15.9
10—CHO—H ₄ F	4	101	5.3	6	74*	18.2	5	78*	16.7
H ₄ F	4	102	4.8	6	85*	16.4	5	80*	13.8
5—CH ₃ —H ₄ F	4	103	10.2	5	121*	17.6	4	88*	3.4

*Significant difference from control (100%) value.

values in N $_2$ O-breathing rats are all significantly higher than in controls. Further, correction of the defect is most effective when formylfolates are added to the incubation mixture. Nevertheless, the results are affected to a significant degree by the inclusion (B) or omission (A) of the additive to the control tubes. This affects both marrow from air-breathing as well as N $_2$ O-breathing animals. Although the addition of folate to the tubes may alter the result it does not change the validity of the dU suppression test which remains either normal or abnormal under both sets of experimental conditions.

The addition of additives to both control and tests results in considerable uniformity of result in air breathing animals (column B) as opposed to the greater range in the absence of the additive (column A).

There were no significant differences in the results of the dU suppression test when this was carried out with and without the additives in the control tubes with normal human marrow, nor with marrow from

FIG. 2. Human marrow cells were incubated with labelled thymidine alone (control) and after the addition of either hydroxocobalamin or folate (F). The results are expressed as a percentage of thymidine uptake in the presence of B $_{12}$ /folate over thymidine uptake alone. The mean $\pm$ s.d. is shown for each group of patients and the number of patients is indicated below each column.

TABLE 4. Comparison of the result of the deoxyuridine suppression test in rat bone marrow with the addition of folate/B $_{12}$ test only (A) and to standard and test (B)

Results as: $\frac{\text{counts with deoxyuridine} + [^3\text{H}] \text{ thymidine} \times 100}{\text{counts with } [^3\text{H}] \text{—thymidine}}$											
Air-breathing rats						N $_2$ O-breathing rats					
Additive	No.	A Mean	s.d.	No.	B Mean	s.d.	No.	A Mean	s.d.	No.	B Mean
Nil	40	7.3	1.59				21	15.7	2.46		
OH—Cbl	21	7.7	1.54	8	6.8	1.62	14	14.1	1.94	8	15.0
5—CHOH $_4$ F	21	7.6	1.42	10	7.5	1.66	16	9.4	1.69	10	9.2
H $_4$ F	14	5.6	1.18	8	6.5*	0.89	12	11.1	1.89	8	12.8*
10—CHOH $_4$ F	8	5.2	1.35	8	6.2	1.22	8	8.6	1.62	8	9.4
5—CH $_3$ H $_4$ F	14	8.3	1.74	8	6.3*	1.55	12	16.9	3.24	8	14.5
F	8	7.5	1.39	4	6.7	1.17	8	14.0	1.83	4	14.7
OHCbl + 5CHOH $_4$ F	14	7.7	1.76	8	7.5	1.20	12	9.2	1.84	8	8.9
OHCbl + H $_4$ F	14	3.0	1.03	8	7.4*	1.33	12	7.5	3.39	8	13.5*
OHCbl + 10CHOH $_4$ F	8	4.7	1.47	8	7.4*	1.26	8	9.9	3.47	8	12.6*
OHCbl + 5CH $_3$ H $_4$ F	14	8.2	1.73	8	6.6*	1.67	11	16.7	3.16	8	14.1
OHCbl + F	6	7.5	1.34	4	7.2	1.89	8	13.1	1.64	4	13.8

*Significant difference between values for A and B.

patients with megaloblastic anaemia due to B_{12} deficiency (Fig. 4) or to folate deficiency (Fig. 5). It is difficult to say whether variation in the results in individual patients under the 2 sets of conditions could be due to such a factor. It would be scientifically more valid to include the additive in the control.

FIG. 3. The results of the dU suppression tests in normal rat marrows and in marrows from rats in whom cobalamin had been inactivated by inhaling a mixture of oxygen and nitrous oxide (1/1). In the tests shown as a filled circle, folate or cobalamin was not added to the control tubes which had marrow and thymidine only. In the tests shown by the unfilled circle, folate and/or cobalamin were added to control tubes as well as to tubes containing first dU and then 3H -thymidine.

Discussion

The effect of folates and cobalamins on the uptake of labelled thymidine may be complex and an attempt to account for these findings in terms of the known pathways involving these cofactors may be an oversimplification (Pelliniemi and Beck, 1980). Nevertheless, in patients with such deficiencies, correction of the defect must play a significant role. The results are least complex in human marrow because the additives had no demonstrable effect on human marrow from haematologically normal subjects. Nor did the addition of hydroxocobalamin to either B_{12} -deficient or folate-deficient marrow (in the absence of added dU) have any significant effect. All the folate additives produced significant

FIG. 4. The results of the dU suppression test in human marrow from patients with megaloblastic anaemia due to cobalamin deficiency. In the tests indicated by the filled circles cobalamin or folate was not added to controls. In the tests indicated by the unfilled circle, folate and/or cobalamin was added to all tubes including controls.

FIG. 5. The results of the dU suppression test in human marrow from patients with megaloblastic anaemia due to folate deficiency. In the tests indicated by the filled circle, cobalamin or folate was not added to controls. In the tests indicated by the unfilled circle, folate and/or cobalamin was added to all tubes including controls.

effects on the utilization of exogenous thymidine. The formylfolates and tetrahydrofolates all reduced the amount of thymidine taken up. In B_{12} deficiency, tetrahydrofolate had a smaller though not statistically significant effect than the other compounds, an effect also noted in the dU suppression test. A plausible explanation for the effect of folates is that they facilitated the methylation of endogenous dU into deoxythymidine and so reduced thymidine requirements.

However, the result of the dU suppression test remained valid despite adding folates to controls. This implies that the methylation of endogenous dU even after addition of the cofactor, was insufficient to meet thymidine requirements in megaloblastic marrows. Possibly the pool of endogenous dU was reduced and exogenous dU, as added in the dU suppression test and within the time allowed for incubation, was not methylated as efficiently to thymidine, as was the case in normal marrows.

The results following the addition of methylfolate is of particular interest because while it reduced the uptake of thymidine in folate-deficient marrows it increased the uptake in cobalamin-deficient marrows. Indeed, if any more procedures were needed to differentiate B_{12} and folate deficiency, these observations would form the basis for such a test. On the same premise methylfolate prevented the methylation of endogenous dU. The methyl group of methylfolate can only be donated to homocysteine to form methionine and itself cannot serve as the methylating agent for dU. Nor does it control the enzyme, methylenetetrahydrofolate reductase, which forms methylfolate from methylenefolate and hence may influence the availability of the single carbon unit donor to deoxyuridine. This step is controlled by the level of S-adenosylmethionine (Kutzbach and Stokstad, 1967). Another hypothetical explanation for the effect of methylfolate is that it blocks folate receptors on the cell surface and prevents transport of folate across cell membranes. Cobalamin deficiency and cobalamin inactivation by N_2O , both interfere with tissue uptake of folates. The mechanism of this is unexplored as yet but gross abnormalities in red cell membranes in megaloblastic patients have been described (Ballas, 1978).

The results with rat marrows are more complex since essentially the same pattern of results appeared with normal and with N_2O -treated marrows. Thymidine uptake was decreased significantly by both 10-formylfolate and tetrahydrofolate. If availability of folate was a limiting factor in haemopoiesis in normal rats then this would account for the similarity in pattern of results in control and N_2O -treated groups. Indeed this may be the case. In both groups, the addition of methyltetrahydrofolate increased the use of exogenous thymidine

significantly, an effect noted only in B_{12} -deficient marrow in man. A possible explanation has been mentioned.

Wickramasinghe and Saunders (1979) have suggested that the omission of the additive in the control tube in the dU suppression test is a defect in its design. While in agreement with this viewpoint it should also be stated that such an addition did not make a significant difference to the results with human material. The results with this test in megaloblastic anaemia are so satisfactory that it would have been surprising to find a major anomaly in its design. Nevertheless there may be marginal differences in some individual cases. The advantage of fully matched controls is evident in the results with rat marrows. With marrows from healthy rats the use of matched controls brought all the mean values with normal marrows to within a very narrow range and this is the justification for using this base in interpreting data in N_2O -treated animals. Unlike the test with human marrow, comparing matched and unmatched control resulted in significant differences in the result when tetrahydrofolate, 10-formyltetrahydrofolate and 5-methyltetrahydrofolate were added in the performance of the dU suppression test.

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