Abnormal Deoxyuridine Suppression Test in Congenital Methylmalonic Aciduria-Homocystinuria Without Megaloblastic Anemia: Divergent Biochemical and Morphological Bone Marrow Manifestations of Disordered Cobalamin Metabolism in Man

By Ralph Carmel and Stephen I. Goodman

We studied two brothers (J.R. and M.R.) with the cobalamin D variant of congenital methylmalonic aciduria-homocystinuria, whose previously reported lack of megaloblastic anemia conflicted with current concepts of cobalamin's role in DNA synthesis and the "methyltetrahydrofolate (MTHF) trap" hypothesis. Both subjects were indeed hematologically normal, although J.R. had a mean corpuscular volume of 96 fl. However, both demonstrated abnormalities in the deoxyuridine suppression test. J.R. had an abnormal suppression value of 21.0% (normal < 10%) that was correctable by adding hydroxocobalamin or folic acid in vitro but not MTHF. M.R. had normal suppression (8.9%), but demonstrated worsening (18.6%) when MTHF was added. J.R.'s classical deoxyuridine suppression pattern of cobalamin deficiency thus supports the trap hypothesis. However, his lack of comparable morphological changes suggests that impaired de novo thymidylate synthesis and the trap hypothesis, though valid, may not fully account for the megaloblastic maturation accompanying cobalamin deficiency. Equally noteworthy was the deleterious effect of MTHF on M.R.'s marrow, suggesting its potential usefulness as an in vitro "stress test" for latent cobalamin abnormality.

MEGALOBLASTIC ANEMIA is the hallmark of cobalamin deficiency in man, but why it happens is not entirely clear. In animals, cobalamin deficiency has yet to produce megaloblastic anemia, even though neurologic abnormalities occur readily; folate deficiency, in contrast, produces such anemia. While species differences may explain that discrepancy, the reported lack of megaloblastic anemia in some children with methylmalonic aciduria-homocystinuria\(^1\)\(^2\) presents somewhat greater difficulty. In this disorder, cobalamin is not converted normally to its two active coenzymes, methylcobalamin and adenosylcobalamin.\(^3\)

The most plausible role of cobalamin deficiency in megaloblastic anemia is described by the "methyltetrahydrofolate (MTHF) trap" hypothesis. Stated briefly, the hypothesis postulates that cobalamin deficiency traps folic acid in its MTHF form because the enzyme mediating the demethylation and recycling of MTHF is cobalamin-dependent. Such trapping depletes the methylene tetrahydrofolate needed for de novo thymidylate synthesis, thereby impairing DNA synthesis. Data accumulated thus far fit this hypothesis better than any other,\(^4\) but acceptance has not been universal, and alternate or supplementary mechanisms have been suggested.\(^5\)\(^6\)

A particularly useful test in demonstrating the defective thymidylate synthesis in megaloblastic anemia and specifically identifying the responsible deficiency has been the deoxyuridine suppression test.\(^7\) This test arose from the observation by Killmann\(^8\) that preincubation of bone marrow cells with deoxyuridine normally suppresses subsequent incorporation of tritiated thymidine into DNA. With megaloblastic cells such suppression is compromised, apparently because the deoxyuridine cannot be converted to thymidylate when methylene tetrahydrofolate is unavailable. Metz et al.\(^7\) expanded this concept, demonstrated its compatibility with the trap hypothesis, and developed it into a diagnostic tool for folate or cobalamin deficiency. Although the mechanisms responsible for the results of this test have been questioned and other effects of the added deoxyuridine on thymidine metabolism demonstrated,\(^5\)\(^9\) the test has proved to be a reliable way to identify megaloblastosis.\(^10\)\(^12\)

Recently we provided deoxyuridine suppression data supporting the trap hypothesis in a child with the cobalamin C mutation variant of methylmalonic aciduria-homocystinuria who had megaloblastic anemia.\(^13\) We now undertook similar studies in two brothers with the cobalamin D mutation who did not have such anemia.\(^2\) The defect in both cobalamin C and D mutations is thought to be a failure to reduce the cobalt in cobalamin from +3 to +1, but is poorly defined. In general, the cobalamin C genetic defect produces a more severe disorder than the cobalamin...
The present studies demonstrate an unusual dissociation between abnormal deoxyuridine suppression test results and the lack of morphological megaloblastosis in man.

CASE HISTORIES

The clinical histories of the two brothers before 1969 have been reported previously. In brief, methylmalonic aciduria and homocystinuria were discovered in J.R. when he was hospitalized for acute psychosis at the age of 14. M.R. was diagnosed at the age of 1 yr when all family members were tested for the metabolic disorder. Initial studies suggested a defect in the biosynthesis of methylcobalamin and adenosylcobalamin, and this has since been confirmed in their fibroblasts. Complementation studies on cultured fibroblasts placed the subjects in the cobalamin D mutant group of methylmalonic aciduria.15

J.R. is now 26 yr old and mildly retarded. He developed an abnormal gait at the age of 15, and is now unable to lift his feet when walking. The cause of this disorder is unknown. Several electromyograms and a biopsy of the vastus lateralis muscle examined by light microscopy have been normal. However, motor (peroneal nerve) and sensory (sural nerve) conduction velocities are decreased.

Left femoral vein thrombosis when he was 18 yr old responded to heparin. Oral betaine hydrochloride (4 g/day) was begun to try to augment the methylation of homocysteine by betaine methyltransferase. He has taken betaine since then but, despite an apparent lowering of his homocysteine excretion, has suffered three more thromboembolic episodes. The first was a left calf vein thrombosis 6 yr ago. Four years ago he developed a thrombosis in the right popliteal area, documented by venography and treated with anticoagulants. Shortly afterwards, while taking acetylsalicylic acid and dipyridamole, he suffered an infarct at the base of the right lung. Since then he has been maintained on warfarin and has had no further symptoms of thrombosis or embolism. He also is receiving carbamazepine. The effect of all in vitro additions on deoxyuridine suppression was always compared to their effect on thymidine incorporation in the absence of the deoxyuridine. The results were expressed in percentages, comparing the deoxyuridine-suppressed tritiated thymidine incorporation with the matched nonsuppressed incorporation. Normally suppressed incorporation is 10% or less.

Serum-mediated uptake of $^{35}$Co-labeled cyanocobalamin by reticuloocyte-rich red cell suspensions, cobalamin levels, folate levels, cobalamin-binding capacity, and urinary excretion of methylmalonic acid and homocystine were measured by methods cited in a previous report.16

RESULTS

Hematologic and Blood Chemistry Data

Both brothers had normal blood counts on admission to the Clinical Research Center. Their peripheral blood smears were normal. J.R. had a red blood cell mean corpuscular volume (MCV) at the upper limits of normal, as he did in 1979 while receiving hydroxocobalamin. Sizing of red blood cells with the Coulter Channelizer (Coulter Electronics, Hialeah, Fla.) done by Dr. Cage Johnson failed to reveal abnormally-sized subpopulations in either brother's blood. Neither subject had oval macrocytes or hypersegmentation of neutrophil nuclei. Both had normal "Arneth counts" (2.60 and 2.82 nuclear lobes/neutrophil). Bone marrow aspirates obtained 92 and 90 days after their last hydroxocobalamin injection

<table>
<thead>
<tr>
<th>Table 1. Hematologic Data</th>
<th>J.R.</th>
<th>M.R.</th>
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</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>14.1</td>
<td>15.4</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>92</td>
<td>98</td>
</tr>
<tr>
<td>Reticulocyte count (%)</td>
<td>1.3</td>
<td>0.6</td>
</tr>
<tr>
<td>WBC/μl</td>
<td>6900</td>
<td>9000</td>
</tr>
<tr>
<td>Platelets x 10⁷/μl</td>
<td>470</td>
<td>360</td>
</tr>
<tr>
<td>Serum cobalamin (ng/liter)</td>
<td>766</td>
<td>560</td>
</tr>
<tr>
<td>Serum folate (μg/liter)</td>
<td>17.2</td>
<td>5.8</td>
</tr>
<tr>
<td>RBC folate (μg/liter)</td>
<td>320</td>
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</tbody>
</table>

*While receiving hydroxocobalamin.
†Three months after discontinuing hydroxocobalamin therapy.
showed no megaloblastic changes in either case. In J.R.'s marrow occasional late polychromatophilic normoblasts had slightly open chromatin, but most cells appeared entirely normal. No abnormalities were seen in the white blood cell series. The morphological interpretations were confirmed independently by two other hematologists, who examined both subjects' smears together with a control smear without knowledge of the patients' identities, and by a hematopathologist. All interpreted the maturation to be normoblastic overall.

Serum cobalamin, serum folate, and red blood cell folate levels were normal, except for the elevated serum folate level in M.R. (Table 1). Neither brother had deficiency of transcobalamin II. M.R. had moderately elevated unsaturated cobalamin-binding capacity (2380 ng/liter) and transcobalamin II (1833 ng/liter). Both subjects' sera mediated cyanocobalamin uptake by reticulocytes adequately (2.7 and 2.8 times the uptake mediated by buffer alone; control values with normal sera ranged between 2.2 and 3.5).

Both brothers had normal blood electrolytes, bilirubin, and ferritin levels. J.R. had a normal lactate dehydrogenase level, but M.R.'s was minimally elevated at 533U/liter (normal 200–500). Both subjects had slightly elevated serum alkaline phosphatase levels. Immunologic function was grossly normal as determined by normal serum immunoelectrophoresis and reactivity to skin testing with mumps antigen. J.R.'s coagulation screening tests were abnormal because he was taking warfarin, while M.R. had normal Quick time, partial thromboplastin time, and thrombin time. Both had normal antithrombin III and β-thromboglobulin levels.

Urinary methylmalonic acid excretion was 921–1490 μg/mg creatinine for J.R. over the 4 days he was studied, and 418–460 μg/mg creatinine for M.R. Both subjects took betaine throughout the study, and their excretion of homocysteine was 0.038–0.109 and 0.008–0.017 μmoles/mg creatinine, respectively. Methylmalonic acid and homocysteine are normally undetectable in urine with the methods used.

**Deoxyuridine Suppression Test Results (Table 2)**

J.R. demonstrated abnormal deoxyuridine suppression. As is classical for cobalamin deficiency, the abnormality was fully corrected by in vitro addition of hydroxocobalamin or folic acid but not by MTHF. M.R. had normal deoxyuridine suppression, but suppression became abnormal when MTHF was added in vitro.

Several control subjects' results are included in Table 2 for comparison. Pernicious anemia subject No. 1 is particularly noteworthy. He was an elderly man with severe subacute combined degeneration and a low serum cobalamin level (44 ng/liter). Like the

| Table 2. Deoxyuridine Suppression Test Results in J.R., M.R., and Several Controls* |
|------------------|------------------|------------------|------------------|------------------|
|                  | Baseline         | Hydroxocobalamin | Folic Acid       | MTHF             |
| In Vitro Additives |                  |                  |                  |                  |
| J.R.             |                  |                  |                  |                  |
| 21.0 [114]       | 8.8 [110]        | 7.0 [86]         | 17.9 [278]       |
| 543              | 1,244            | 1,224            | 1,553            |
| M.R.             |                  |                  |                  |                  |
| 8.9 [291]        | 7.5 [296]        | 7.7 [319]        | 18.6 [657]       |
| 3.277            | 3,933            | 4,118            | 3,525            |
| Pernicious anemia No. 1 | 43.5 [698] | 16.5 [433] | 16.6 [491] | 30.2 [901] |
| 1,160            | 2,630            | 12,958           | 2,988            |
| Pernicious anemia No. 2 | 43.6 [1,338] | 29.0 [872] | 24.3 [947] | 40.5 [1,477] |
| 3,067            | 3,005            | 3,889            | 3,649            |
| Folate deficiency | 38.9 [2,102]    | 35.0 [2,155]     | 10.5 [495]       |
| 5,405            | 6,159            | 4,720            |
| Normoblastic anemia No. 1 | 4.5 [256] | 4.1 [232] | 3.2 [239] | 6.5 [501] |
| 5,650            | 5,602            | 7,831            | 7,717            |
| Normoblastic anemia No. 2 | 2.2 [98] | 2.3 [127] | 3.0 [152] | 5.098 |
| 4,506            | 5,601            | 5,098            |

*Results are given as percent residual tritiated thymidine incorporation after adding deoxyuridine. In parentheses are the actual data (the average of triplicates) in counts per minute per 3 x 10⁶ nucleated cells; the numerator is the deoxyuridine-suppressed value and the denominator is the matched deoxyuridine-free control.

†A value of 17.5% (82/468) was obtained using a different tritiated thymidine batch in a concurrent experiment.

†A value of 6.9% (259/3,743) was obtained using a different tritiated thymidine batch in a concurrent experiment.

§Cyanocobalamin was used instead of hydroxocobalamin in this experiment.
two brothers, he presented with a normal hemoglobin level (15.1 g/dl). He excreted 218 µg methylmalonic acid and 0.022 µmole homocystine/mg creatinine. He had mild macrocytosis (MCV 101 fl). Unlike the two brothers, however, his bone marrow and peripheral blood smears showed unmistakable megaloblastic changes. His deoxyuridine suppression test was strikingly abnormal. Also noteworthy is pernicious anemia subject no. 1 had slight improvement, but Table 2 shows that the improvement was poor compared with that induced by folic acid. Among normoblastic marrows there was occasional minimal worsening by MTHF, but the effect was not consistent, and even when present, was so small as to be within the range of experimental variability.

DISCUSSION

As noted originally,2 neither of the two brothers had megaloblastic anemia. In fact they were not even anemic. Although J.R. had an MCV at the upper limits of normal and macrocytosis precedes the megaloblastic changes in bone marrow,17 the MCV was unchanged from 1979 when he was receiving hydroxocobalamin, and neither his marrow nor his peripheral blood demonstrated any clear morphological evidence of megaloblastosis. Despite this, his marrow cells showed abnormal deoxyuridine suppression. The abnormality was corrected by hydroxocobalamin or folic acid but not by MTHF, a pattern classical for cobalamin deficiency.7 M.R.’s marrow cells had normal suppression, but did demonstrate a subtle abnormality, namely a striking impairment of deoxyuridine suppression with the in vitro addition of MTHF.

The reason for the different behavior of the two marrows is not clear, but is most easily explainable by assuming that tissue cobalamin status in M.R. was better than in J.R. Although we did not measure the tissue cobalamin levels, the lower methylmalonic acid excretion by M.R. supports this contention. M.R. was devoid of all clinical manifestations of the disorder. However, it is also worth noting that he is only now reaching the age when his older brother first developed noticeable clinical difficulties. The disorder may well have a relatively long subclinical period.

The bone marrow findings lead to several conclusions. The abnormal deoxyuridine suppression pattern with the typical in vitro responses in a patient heretofore thought to have normal bone marrow despite his cobalamin problem strengthens the validity of the MTHF trap hypothesis. It seems highly unlikely that our results reflect a defect in deoxyuridine metabolism unrelated to the trap hypothesis in view of the classical responses to the in vitro additives. At the same time, the lack of appropriate megaloblastic morphological changes implies that a block at the step measured by
the deoxyuridine suppression test, while a valid marker of cobalamin or folate deficiency, is not by itself sufficient to cause megaloblastic anemia, and that additional mechanisms combine in acquired cobalamin deficiency and in the cobalamin C form of methylmalonic aciduria-homocystinuria to produce the hematologic changes.

Several other explanations for the discrepancy we describe are possible. Perhaps, the thymidylate defect is usually sufficient for full expression, but the two brothers have a mild, perhaps "leaky" metabolic block or their bone marrow is relatively spared by an unequal susceptibility of different tissues. An alternative possibility is that the morphological sequelae could be masked by a coexisting problem, such as occurs with iron lack. However, when coexisting iron lack (not a factor here) blunts megaloblastic changes, it blunts the deoxyuridine suppression abnormality equally. There is no precedent for coexisting disorders causing dissociation between these two manifestations.

Whatever the explanation, the morphological contrast between J.R. and pernicious anemia patient no. 1, who had a similar blood count and MCV, is striking. It is interesting to note in this context that, like J.R., animals made cobalamin deficient have the classical abnormalities in the deoxyuridine suppression test yet never develop megaloblastic anemia.

Further study of cobalamin-deficient animals may help unravel this puzzle. The specifics of the DNA abnormality leading to megaloblastic proliferation remain elusive, even if the trap hypothesis is accepted in its entirety. For example, the salvage pathway for thymidylate synthesis should compensate for the impaired de novo pathway and megaloblastic cells in fact appear to have normal thymidylate content. Various additional mechanisms have been suggested.

Several of our observations are of further interest. J.R. actually demonstrated quite low thymidine incorporation into DNA even without deoxyuridine (Table 2). We do not know the reason nor if that has anything to do with his lack of megaloblastosis. Another technical observation is our previously emphasized need for incorporating a deoxyuridine-free control for each additive, rather than relying on a single baseline control for the entire experiment. Table 2 illustrates the often unpredictably great variability in thymidine incorporation induced by different additives within the same marrow.

Finally, the deleterious effect of MTHF may have potential utility in studying cobalamin deficiency. Tables 2 and 3 show how M.R.'s otherwise normal deoxyuridine suppression was impaired by MTHF added in vitro and how this also occurred in a previously studied patient with this syndrome even after hematologic remission was induced and her deoxyuridine test had become normal. In contrast, patients with acquired cobalamin deficiency in our study and in previous studies did not manifest such a worsening; they merely failed to demonstrate correction by MTHF. Deacon et al. have suggested that even normal marrows may be slightly worsened by MTHF, but our data and that of others in normal controls do not bear this out.

The inhibitory effect of MTHF on thymidylate synthesis in methylmalonic aciduria-homocystinuria is therefore intriguing. Perhaps it acts in some way as a competitive inhibitor of more readily utilizable folates. It may also constitute a useful "stress test" to uncover other subtle or latent cobalamin deficiency states. Preliminary studies have identified several such potential cases (Carmel R: unpublished data), and further investigation of this phenomenon is now under way.

The accumulated evidence now allows us to conclude that virtually all patients with methylmalonic aciduria-homocystinuria have "biochemical megaloblastosis," probably even those few who do not have clear morphological megaloblastosis. Our two brothers exemplify the latter disparity and can be regarded as having a milder cobalamin defect. J.R. had a fully abnormal deoxyuridine suppression. It may also be noted that his defect was fully correctable with hydroxocobalamin, as is seen in acquired cobalamin deficiency but which contrasts with our inability to correct the abnormal results even with methylcobalamin in the child with the cobalamin C mutation. M.R. may be said to have had a defect so subtle that it could be brought out only by adding MTHF.

These cases of congenital methylmalonic aciduria-homocystinuria may provide clues to the still elusive mechanisms of megaloblastic anemia. The demonstrated thymidylate synthetic defect that results from methylene tetrahydrofolate unavailability and clearly seems cobalamin-related may lead to slowed DNA synthesis and result only in macrocytosis at best. Perhaps, additional mechanisms usually leading to the characteristic nuclear morphological changes remain unaffected and the full hematologic expression of
megaloblastosis never develops. The result is in contrast to most other patients with this syndrome and all patients with acquired cobalamin deficiency, but resembles in many respects the picture in cobalamin-deficient animals.

REFERENCES


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