Significance of subnormal red-cell folate in thalassaemia

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SYNOPSIS  Subnormal red-cell folate values were encountered in 20 of 63 thalassaemic subjects in a population with a low incidence of megaloblastosis. The abnormality was not associated with haematological changes of megaloblastosis and could not be explained on the basis of incomplete liberation of intra-erythrocytic folates, serum conjugase deficiency or vitamin B₁₂ deficiency. Evidence is presented to indicate that it is unlikely to represent a state of subclinical folate deficiency. The exact cause of the abnormality is unknown.

In the absence of frank megaloblastosis, the diagnosis of folate deficiency is not straightforward. Hypersegmentation of circulating neutrophils is neither a specific (Chanarin et al, 1965) nor a sensitive (Hibbard and Hibbard, 1971) index. Increased urinary formiminoglutaric acid excretion after histidine load is found not only in folate deficiency but also in vitamin B₁₂ deficiency (Knowles and Prankerd, 1962; Chanarin, 1964), chronic liver disease (Carter et al, 1961) and other disease states (Gräsbeck et al, 1961; Kershaw and Girdwood, 1964; Mohamed and Roberts, 1966). Serum folate is largely maintained by an exchange between dietary folic acid and the storage folate (Chanarin and McLean, 1967) and may become reduced with relatively short periods of dietary deprivation (Herbert, 1962) even in the presence of normal stores. Folates apparently gain entry into the red cell only at the stage of the marrow erythroblast but do not enter mature red cells in the circulation (Herbert and Zalusky, 1962; Neal and Williams, 1965). Since red cells do not lose folate during their life span in the peripheral blood (Hansen and Weinfeld, 1962), the folate content of circulating red cells will reflect the availability of folate some weeks earlier, at a time when they were being formed in the marrow. Where short-term fluctuations do not occur, red-cell folate is considered to be a useful index of the folate status.

So far the factor other than a low body folate status which may reduce red-cell folate is vitamin B₁₂ deficiency (Cooper and Lowenstein, 1964; Hoffbrand et al, 1966). In the course of a study on the blood folate values in thalassaemic subjects in the steady state, a number of patients were found to have subnormal red-cell folate levels. The purpose of this communication is to report these results and to show that the abnormal finding was neither a technical artefact nor due to vitamin B₁₂ or folate deficiency. It is concluded that yet another factor or factors may affect the red-cell folate level and that they may be related to the thalassaemic state.

Material and Methods

PATIENTS
Sixty-three thalassaemic subjects in the steady state, with normal nutrition, having no intercurrent illness and not on any medication, were studied (table I). There were 34 males and 29 females, none of whom was pregnant or on oral contraceptives within the six months before the study. All were iron-sufficient, as shown by normal serum iron and the presence of stainable iron in marrow aspirates. None had hypersegmentation of neutrophils in the peripheral blood. Bone marrow biopsy was performed.

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formed in 11 and all showed normoblastic haemopoiesis.

Standard haematological methods were employed (Dacie and Lewis, 1968). Methods for the determination of red cell volume and red cell survival have been described previously (Tso, 1972). Where the effect of folic acid therapy was studied, pteroylglutamic acid, 10 mg per day, was given orally in two divided doses for at least four weeks. Blood for folate studies was repeated three to seven days after cessation of therapy.

FOLATE AND VITAMIN B12 ASSAYS
Fasting blood samples were used. Serum and red-cell folates were assayed microbiologically with Lactobacillus casei (ATCC 7469), employing the methods of Waters and Mollin (1961) and Hoffbrand et al (1966). The control values for the population were 19 to 20-5 µg/l (mean 6-3, standard deviation 3-6) for serum and 155 to 600 µg/l (mean 295, standard deviation 120) for red cells. Serum vitamin B12 was assayed with Lactobacillus leichmannii (ATCC 8118), using the method of Matthews (1962).

IN VITRO STUDIES
Freeze-thaw Lysate
Whole blood anticoagulated with EDTA from each of eight thalassaemic patients was divided into two aliquots; one was haemolyzed in 9 volumes of 1% ascorbic acid and the other was thrice frozen in acetone/solid CO2 mixture and thawed. The freeze-thaw lysate so prepared was then added to 9 volumes of 1% ascorbic acid as for whole blood. Both specimens from the same patient were assayed for folate in the same batch.

Conjugase Activity
A control group O whole blood specimen in EDTA was divided into two aliquots; the first was processed for red-cell folate assay, and the other was centrifuged to remove serum, washed thrice with cold isotonic saline, and further divided into several fractions. Each was then suspended in about 2 volumes of a thalassaemic serum, the conjugase activity of which was to be determined. The haematocrit of each suspension was measured and the specimens were processed for red-cell folate assay in the same batch.

Results
In this laboratory the lower limits of normality for serum and red-cell folates were taken as 2 µg/l and 155 µg/l. Table II shows that subnormal levels were observed in the serum folate in 16 patients (25-4%) and in the red-cell folate in 20 (31-7%). Only 12 (19-0%), however, had subnormal values in both parameters. An apparently higher frequency of abnormality was seen in Hb E-thalassaemia and β-thalassaemia major and a lower one in β-thalassaemia minor, but these are not statistically significant.

Of the 20 with subnormal red-cell folate values, 11 were males (33-3%) and 9 females (30-0%). The serum B12 level was normal in 11 in whom this was measured. Bone marrow aspirate was obtained in 10 and all showed active normoblastic erythropoiesis, the presence of stainable iron, and no giant metamyelocytes.

Among patients with haemoglobin H disease, the largest single variety of thalassaemia in the present study, there was no statistical difference in haematological values between those with normal and those with subnormal red-cell folate levels (table III). The figure further shows the lack of statistical correlation between the individual red-cell folate values and the red-cell survival in these patients. It is particularly noteworthy that in two patients, whose red-cell survival improved after splenectomy, red-cell folate values in fact showed a fall.

Folic acid therapy was started in 11 patients with subnormal red-cell folate after excluding vitamin B12 deficiency. None showed any rise in reticulocyte count or in the haemoglobin level though in all six in whom the red-cell folates were repeated there was correction of the abnormality.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number Studied</th>
<th>Number (percentage) with Subnormal Folate Value in Serum and Red Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb H disease</td>
<td>33</td>
<td>6 (18-2) 11 (33-3) 5 (15-2)</td>
</tr>
<tr>
<td>Heterozygous α-thalassaemia</td>
<td>14</td>
<td>5 (35-7) 3 (21-4) 2 (14-3)</td>
</tr>
<tr>
<td>β-thalassaemia minor</td>
<td>7</td>
<td>0 (0) 0 (0) 0 (0)</td>
</tr>
<tr>
<td>Hb E-thalassaemia</td>
<td>5</td>
<td>3 (60-0) 3 (60-0) 2 (40-0)</td>
</tr>
<tr>
<td>β-thalassaemia major</td>
<td>4</td>
<td>2 (50-0) 3 (75-0) 3 (75-0)</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>16 (25-4) 20 (31-7) 12 (19-0)</td>
</tr>
</tbody>
</table>

Table II Frequency of abnormal folate values related to diagnosis

<table>
<thead>
<tr>
<th>Diagnoses</th>
<th>Normal RBC Folate</th>
<th>Subnormal RBC Folate</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9-2 ± 2-0 (11)</td>
<td>9-8 ± 1-4 (5)</td>
<td>&gt; 0-5</td>
</tr>
<tr>
<td>Female</td>
<td>8-5 ± 1-2 (12)</td>
<td>7-9 ± 2-0 (6)</td>
<td>&gt; 0-6</td>
</tr>
<tr>
<td>Red-cell volume (ml/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28-4 ± 0-0 (6)</td>
<td>28-7 ± 3-5 (2)</td>
<td>&gt; 0-8</td>
</tr>
<tr>
<td>Female</td>
<td>21-8 ± 3-3 (5)</td>
<td>26-5 ± 4-1 (4)</td>
<td>&gt; 0-1</td>
</tr>
<tr>
<td>Red-cell survival (T4 Cr in days)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>14-8 ± 4-3 (11)</td>
<td>15-8 ± 4-1 (6)</td>
<td>&gt; 0-6</td>
</tr>
</tbody>
</table>

Table III Haemoglobin H disease: relation of haematological values to red-cell folate

Figures represent mean ± standard deviation (number of observations in parenthesis)
The results of the in vitro studies are presented in tables IV and V. It can be seen from table IV that in all eight thalassaemic specimens studied, the availability of red-cell folate to the assay organism was the same whether lysis was achieved by ascorbic acid or by freeze-thawing. Particularly in specimens 7 and 8, where subnormal red-cell folate levels were detected using the conventional method of lysis, no increase was found with freeze-thawing. From table V it is seen that in two separate experiments no difference could be demonstrated in the serum conjugase activities between thalassaemic patients with normal red-cell folate and those with subnormal values.

Discussion

Megaloblastic anaemia is an uncommon condition among the Chinese in Hong Kong. Among some 35,000 admissions to the University Department of Medicine at Queen Mary Hospital over a 10-year period, there were 15 patients with megaloblastic anaemia, of which only three were due to folate deficiency. Thus the finding of subnormal red-cell folate in 51.7% of a random sample of thalassaemic subjects is of great interest. The method of Hoffbrand et al. (1966) for the estimation of red-cell folate has been evaluated as providing the optimum conditions for the maximal yield in healthy subjects (Omer, 1969), but its efficacy in thalassaemia remains unknown. The present data show that while thalassaemic red cells are more resistant to osmotic lysis even to a tonicity of 30 mOsm/kg (Weatherall and Clegg, 1972), the mixing at room temperature of 9 volumes of 1% ascorbic acid (pH 2.6; 55 mOsm/kg) to 1 volume of thalassaemic whole blood (final mixture: pH 3.7, 68 mOsm/kg) is as effective in liberating intra-erythrocytic folate as freeze-thawing. Though little is known of the inheritance of serum conjugase (\( \gamma \)-glutamyl peptidase) in the human, it appears unlikely from the present results that a deficiency in this enzyme is responsible for the subnormal red-cell folate in thalassaemia.

The findings of Herbert (1962) suggest that megaloblastosis is a late manifestation of folate deficiency. Furthermore, it has been postulated that the deficient haemoglobin synthesis in red cells in thalassaemia might render the morphological recognition of megaloblasts difficult (Chanarin, 1969). Since vitamin B12 deficiency has been excluded in at least half of the patients, the possibility still exists that the subnormal red-cell folate in the present series of thalassaemic subjects represented a subclinical deficiency state even though erythropoiesis was normoblastic in all 11 marrows studied. This, however, appears unlikely for several reasons.
First, in the previously reported cases of folate deficiency in thalassaemic syndromes associated with normoblastic erythropoiesis, there was evidence of either marrow hypoplasia or megaloblastic granulopoiesis and all showed haematological response to folate therapy (Chanarin, 1969; Jandl and Greenberg, 1959; Luhby and Cooperman, 1961). None of these was observed in the present study. Since a rise in red-cell folate value was seen after treatment, malabsorption or aberrant metabolism may be excluded. Secondly, there was a lack of correlation between the red-cell folate levels and the degree of anaemia or the severity of haemolysis among thalassaemic patients of a single variety, namely Hb H disease. Thirdly, if the 30% of the female patients in this study were having subclinical folate deficiency, it might be expected that at least a similar proportion of thalassaemic pregnancies would be complicated by folate deficiency but experience in this same population did not bear this out. For instance, Todd and Kan (1965) found no megaloblastosis in 38 thalassaemic pregnancies, and, more recently, Tso and Wong (1975) observed that none of 35 thalassaemic subjects had subnormal red-cell folate during late pregnancy and the puerperium.

It appears from these observations that subnormal red-cell folate may be found in a situation other than folate or vitamin B$_{12}$ deficiency. It is also tempting to conclude that this is in some way related to the thalassaemia state. While the exact mechanism remains to be elucidated, caution must be exercised in interpreting subnormal red-cell folate values in thalassaemia, especially in the absence of frank megaloblastosis.

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References


