Brief Report

Observations on Conjugated and Unconjugated Blood Folate Levels in Megaloblastic Anemia and the Effects of Vitamin B₁₂

By K. N. Jeejeebhoy, S. M. Pathare and J. M. Noronha

The microbiologic assay of serum folates has been commonly used for determining the folate status of patients with megaloblastic anemia. The organism used is Lactobacillus casei and the L. casei activity in serum has been shown to be largely due to N⁵-methyltetrahydrofolate.¹ It is known that the major part of whole blood folate activity resides in the cells² and is several times the amount in serum. Moreover Noronha and Aboobaker² have shown that cellular folates are likely to be conjugated forms of N⁵-methyltetrahydrofolates, the major portion of which becomes available to L. casei only after prior conjugase digestion of blood extract² or incubation of hemolysed cells with plasma enzymes.³

In vitamin B₁₂ deficiency an increase in serum L. casei activity has been observed,⁴ the cause of which had remained speculative. Since the major part of whole blood folates, especially cellular folates, are known to be conjugated, it is obvious that a complete picture of the effect of vitamin B₁₂ on blood folates cannot be obtained without a study of both conjugated and unconjugated forms of folate. The results suggest that vitamin B₁₂ may have a fundamental role in the metabolism of cellular conjugated folates.

Material and Methods

Blood Folate Levels

These were estimated on protein-free extracts of whole blood. The extracts were made by adding 1 ml of freshly drawn whole blood to 10 ml of 0.1 M phosphate buffer pH 6 containing 1 per cent ascorbate. Great care was taken to deproteinize the blood immediately after withdrawal to prevent any possible incubation of cellular folates with the plasma. The blood-buffer mixture was heated in a boiling water bath for 15 minutes and centrifuged at 3000 rpm for 10 minutes. The clear supernatant was stored frozen at −20°C until assayed microbiologically.

Unconjugated folates were determined microbiologically on extracts using L. casei. However, it is known that L. casei activity prior to conjugase digestion also represents triglutamyl pteroate derivatives.⁶ This factor is of little consequence, as the major proportion of conjugated folates in human blood is more complex.²

Conjugated folate levels were determined after overnight digestion of blood extract with chicken liver conjugase. This digestion process has been shown to make all con-

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Table 1.—Clinical Features of Patients Studied

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Hb. Gm./100 ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>M</td>
<td>32</td>
<td>Megaloblastic Anemia—Folate Deficiency</td>
<td>Tropical sprue 10.5</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>37</td>
<td>Tropical sprue</td>
<td>8.6</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>68</td>
<td>Nutritional megaloblastic anemia</td>
<td>10.0</td>
</tr>
<tr>
<td>11</td>
<td>N</td>
<td>25</td>
<td>Tropical sprue</td>
<td>11.6</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>34</td>
<td>Megaloblastic Anemia—Vitamin B&lt;sub&gt;12&lt;/sub&gt; Deficiency</td>
<td>Tropical sprue 10.2</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>65</td>
<td>Megaloblastic anemia and gall stones</td>
<td>5.2</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>18</td>
<td>Tropical sprue</td>
<td>8.6</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>48</td>
<td>Nutritional megaloblastic anemia</td>
<td>9.4</td>
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<tr>
<td>16</td>
<td>M</td>
<td>45</td>
<td>Postgastrectomy anemia</td>
<td>10.2</td>
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<td>17</td>
<td>M</td>
<td>40</td>
<td>Tropical sprue</td>
<td>9.7</td>
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<tr>
<td>18</td>
<td>F</td>
<td>15</td>
<td>Tropical sprue</td>
<td>13.0</td>
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<tr>
<td>19</td>
<td>F</td>
<td>40</td>
<td>Tropical sprue</td>
<td>4.5</td>
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<td>20</td>
<td>F</td>
<td>35</td>
<td>Tropical sprue</td>
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<tr>
<td>21</td>
<td>F</td>
<td>8/12</td>
<td>Normoblastic Hemopoiesis with Iron Deficiency</td>
<td>Encephalitis 10.0</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>18</td>
<td>Cirrhosis of liver</td>
<td>8.5</td>
</tr>
<tr>
<td>23</td>
<td>M</td>
<td>42</td>
<td>Partial gastrectomy-anemia</td>
<td>5.5</td>
</tr>
</tbody>
</table>

jugated folates available to *L. casei*. Two tenths ml. of the blood extract was digested with 0.05 ml. chicken liver conjugase in the presence of 1 ml. 0.1 M acetate buffer pH 4.7 at 37 C. under toluene. The conjugase digest was then made to suitable volume in 1 per cent ascorbate to correct for evaporation and subjected to microbiologic assay with *L. casei* as mentioned above. The increase in folate level after conjugase digestion was taken as a measure of conjugated forms of N<sup>5</sup>-methyltetrahydrofolate. The conjugase preparation was initially purified to remove most of the folic acid present and the final product was shown to contain not more than 1 μg. *L. casei* folate activity per ml. Even this insignificant amount of folate activity was corrected for by the use of conjugase blanks. Serum vitamin B<sub>12</sub> was assayed microbiologically using *Euglena gracilis*.7

Subjects Studied

The folate pattern and vitamin B<sub>12</sub> content were estimated in 7 control subjects (Nos. 1 to 7) who were healthy volunteers. 14 patients with megaloblastic anemia (Nos. 8 to 20) and 3 patients with iron deficiency anemia (Nos. 21 to 23). The clinical details of the patients are given in table 1.

Results

Control Subjects

The results are presented in figure 1. The unconjugated folate levels varied between 4.0 and 16.1 μg./ml in blood with a mean value of 9.1 μg./ml. The conjugated folates amounted to about ten times the unconjugated folates and varied between 61.2 to 109 μg./ml. with a mean value of 91.7 μg./ml.
Fig. 1.—Relationship of conjugated and unconjugated folates in subjects studied. Note that normal subjects and patients with iron deficiency anemia occupy the upper right of the figure. The vertical and horizontal lines mark the lower limits of the levels of unconjugated and conjugated folates, respectively in control subjects. Folate-deficient patients are largely restricted to the lower left quadrant and vitamin B_{12}-deficient patients are scattered in the lower right quadrant of the figure.

Patients with Megaloblastic Anemia

There were four patients with normal vitamin B_{12} levels but low unconjugated folates (0.7–3.2 with a mean of 1.9 mg./ml.) indicating that they were folate deficient (fig. 1). The values for conjugated folates were also low and varied between 32 and 91 mg./ml. with a mean of 41.0 mg./ml. In these patients, with folate deficiency, there was a twenty-fold greater concentration of conjugated folates over the unconjugated derivatives.

In patients with vitamin B_{12} deficiency (fig. 1), the unconjugated folate levels were high and varied between 5.7 and 44.5 mg./ml. with a mean value of 26.1 mg./ml. The conjugated folates were, however, low and varied between 11.5 and 50.0 mg./ml. with a mean value of 29.8 mg./ml. Unlike control subjects and folate deficient patients, the average ratio between conjugated and unconjugated folate levels amounted to about 1.3.
VITAMIN B₁₂ EFFECTS IN MEGALOBLASTIC ANEMIA

Fig. 2.—Effect of vitamin B₁₂ on the conjugated and unconjugated folate levels in patients with and without vitamin B₁₂ deficiency. Note that vitamin B₁₂-deficient patients prior to B₁₂ therapy occupied the lower right hand quadrant of the figure. There is a marked shift of these points to the upper left of the figure after injecting 100 µg. of vitamin B₁₂ due to a simultaneous rise in conjugated folates with a fall in unconjugated folates. No such change is seen in patients with normal serum vitamin B₁₂ levels.

Patients with Iron Deficiency Anemia and Normoblastic Erythropoiesis

The results are given in figure 1. The unconjugated folates varied from 6.2 to 7.6 mg./ml. and the conjugated folates varied from 50.4 to 126 mg./ml., the results being very similar to those in control subjects.

Effect of Vitamin B₁₂ on the Blood Folate Patterns

The folate levels were repeated 6 days after a single injection of 100 µg. of vitamin B₁₂. This period coincided with about the peak reticulocyte response to the injection in patients with vitamin B₁₂ deficiency. However at this time the haemoglobin had not risen significantly above the level prior to injection. Following this injection, the blood folate pattern in patients with vitamin B₁₂ deficiency reverted to normal with a fall in unconjugated forms and a rise in conjugated folates (fig. 2). The results in general are
consistent with the findings of Cooperman and Lowenstein who showed that erythrocyte folate deficiency in patients with pernicious anemia was corrected by vitamin B₁₂ therapy.

In contrast, vitamin B₁₂ had no appreciable effect on the folate pattern of 4 patients, 1 with folate deficiency (case 9) and 4 with iron deficiency (cases 21 to 23), all of whom had normal vitamin B₁₂ levels (fig. 2).

**DISCUSSION**

It is obvious from the results that lack of vitamin B₁₂ depletes the cells of conjugated folates which are the main forms of intracellular folates in man. The low level of conjugated folates in an anemic subject may perhaps be explained by a reduction in the mass of erythrocytes. This however is unlikely to be the sole cause of low conjugated folates in patients with vitamin B₁₂ deficiency for the following reasons: firstly, the mean hemoglobin levels in patients with vitamin B₁₂ deficiency and iron deficiency anemia were about the same being 8.1 Gm. per cent and 8.5 Gm. per cent respectively, whereas the mean level of conjugated folates in the former was less than half that in the latter, 29.8 μg./ml. and 78 μg./ml. respectively; secondly, there is a marked change in folate pattern on giving vitamin B₁₂ to deficient patients without any significant rise in hemoglobin, indicating again that the effect was specifically related to the administration of vitamin B₁₂. The specific nature of this relationship was further demonstrated by the negligible effect of vitamin B₁₂ on the folate pattern in patients who were not vitamin B₁₂ deficient.

In folate deficiency, the cells appeared to show an increased avidity for conjugated folates at the expense of unconjugated forms, suggesting that conjugated folates may be metabolically more important.

It has been suggested earlier that in vitamin B₁₂ deficiency, N⁵-methyl-tetrahydrofolic acid piles up because vitamin B₁₂ was considered to be involved in its utilization. This “piling up” could thus reduce the release of *S. faecalis* R. -active tetrahydrofolate required for other 1-C reactions. If this hypothesis was correct, there should have been an increase of such non-methyltetrahydrofolates (*S. faecalis* R. activity) when folate clearance patterns were determined in subjects replete with respect to vitamin B₁₂, for which there is no experimental evidence. Although our experiments confirm the occurrence of elevated monoglutamyl folate in vitamin B₁₂-deficient patients, administration of vitamin B₁₂ to such patients resulted in a spectacular rise of folyl conjugates (fig. 2). Based on these findings, it would seem more plausible to suggest that the block in vitamin B₁₂ deficiency lies in the conversion of monoglutamyl derivatives to conjugated folates.

These hitherto unreported results lead one to speculate that vitamin B₁₂ deficiency results eventually in a relative tissue depletion of conjugated folates. The interrelationship may explain why hemopoietic changes of vitamin B₁₂ deficiency can be reversed partially by giving large amounts of folic acid. If vitamin B₁₂ has a role in maintaining adequate tissue concentrations of the conjugated forms which have been shown to have greater cofactor activity in bacterial systems, it might help explain the known
effect of vitamin B\textsubscript{12} on established folate-dependent reactions related to formiminoglutamic acid\textsuperscript{11} and aminoimidazolecarboxamide\textsuperscript{12} excretions. However, a role for conjugated folates in data obtained from bacterial systems, referred to above has yet to be confirmed in mammals.

**Summary**

Vitamin B\textsubscript{12} deficiency was associated with a rise in unconjugated folates and marked depletion of intracellular conjugated folates. The changes could be reversed by giving vitamin B\textsubscript{12}. These results probably indicate a way by which vitamin B\textsubscript{12} and folic acid are interrelated at the cellular level.

**Summario in Interlingua**

Carentia de vitamina B\textsubscript{12} esseva associate con un augmento del non-conjugate folatos e un marcate depletion de conjugate folatos intracellular. Le alterationes poteva esser revertite per le administration de vitamina B\textsubscript{12}. Il es probabile que iste resultatos indica como vitamina B\textsubscript{12} e acido folic es interrelationate al nivello cellular.

**REFERENCES**

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