Methemoglobinemia and ascorbate deficiency in hemoglobin E β thalassemia: metabolic and clinical implications

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During investigations of the phenotypic diversity of hemoglobin (Hb) E β thalassemia, a patient was encountered with persistently high levels of methemoglobin associated with a left-shift in the oxygen dissociation curve, profound ascorbate deficiency, and clinical features of scurvy; these abnormalities were corrected by treatment with vitamin C. Studies of erythropoietin production before and after treatment suggested that, as in an ascorbate-deficient murine model, the human hypoxia induction factor pathway is not totally dependent on ascorbate levels. A follow-up study of 45 patients with HbE β thalassemia showed that methemoglobin levels were significantly increased and that there was also a significant reduction in plasma ascorbate levels. Haptoglobin levels were significantly reduced, and the high frequency of the 2.2 haptoglobin genotype may place an additional pressure on ascorbate as a free-radical scavenger in this population. There was, in addition, a highly significant correlation between methemoglobin levels, splenectomy, and factors that modify the degree of globin-chain imbalance. Because methemoglobin levels are modified by several mechanisms and may play a role in both adaptation to anemia and vascular damage, there is a strong case for its further study in other forms of thalassemia and sickle-cell anemia, particularly when splenic function is defective. (Blood. 2012;120(15):2939-2944)

Introduction

Although low ascorbate levels have been observed in patients with different hemoglobinopathies,1,2 there are very few reports of the clinical manifestations of scurvy in these conditions.3 During an analysis of the mechanisms for the broad phenotypic diversity of hemoglobin E (HbE) β thalassemia in Sri Lanka, a patient was encountered with profound ascorbate deficiency and clinical features of scurvy who also had a high level of methemoglobin. This unusual combination of findings has raised several important questions. First, to what extent does ascorbate deficiency interfere with the hypoxia-sensing mechanism in humans, particularly with respect to erythropoietin response to anemia? The key players in this pathway are the prolyl hydroxylase domain-containing enzymes that catalyze the prolyl-4-hydroxylation of the hypoxia-inducible factor in the presence of oxygen and 2-oxoglutarate as cosubstrates with iron and ascorbic acid as cofactors.4-6 Recent work in ascorbate-deficient mice suggests that other fail-safe mechanisms are involved in this reaction and that erythropoietin response is not altered in ascorbate deficiency.7,4; is this the case in humans? The second question raised by this unusual patient report is, because of the potential deleterious effects of methemoglobin on a patient’s response to anemia8 and the vascular endothelium,9,10 (1) how common are increased levels of methemoglobin in HbE β thalassemia and related disorders, (2) to what extent might this depend on ascorbate deficiency, and (3) what other factors may be involved?

The results of these studies suggest that, with respect to hypoxia recognition, humans are able to compensate for ascorbate deficiency in the same way as the murine model. There is a highly significant increase in methemoglobin production in HbE β thalassemia and a significant reduction in plasma ascorbate levels, although not to those observed in the patient whose findings initiated this study. There is, however, a highly significant relationship between the level of methemoglobin and splenectomy and also with the factors that modify globin-chain imbalance. A further complication in this population was the finding that the haptoglobin genotype was nearly all of the 2.2 variety, which is less effective at hemoglobin binding11,12 and may place an additional pressure on ascorbate as a free radical scavenger.

Clearly, there are multiple factors involved in the increased level of methemoglobin production in this form of thalassemia and because of its potential effects on adaptation to anemia and vascular damage, further studies of the mechanisms involved in its increased production are required in other types of thalassemia and sickle-cell anemia.

Methods

Patients

The subject in whom the findings initiated this study was a 19-year-old female patient attending the National Thalassemia Center, Kurunegala, Sri Lanka. She had presented at 2 years of age with anemia and splenomegaly, and later the diagnosis of HbE β thalassemia was established. For the next


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7 years, she received intermittent transfusion, after which she underwent splenectomy on the basis of a steady-state hemoglobin level of 5.6 g/dl and a spleen that was enlarged to 19 cm below the costal margin. For the next 10 years, despite hemoglobin values in the 7.8 g/dl range, her growth and sexual maturation were delayed, although by the age of 19 years, she had achieved midparental height and the menarche. Only when she reached the age of 17 years did the patient and her family disclose that she had had painful and enlarged gums and intermittent mucosal bleeding for several years. A detailed dietetic history at this time revealed that she ate no fruit of any form or vegetables, a diet that had persisted for several years. The results of a screening for environmental factors that might induce methemoglobinemia were negative.

On the basis of subsequent findings in this patient and her family, detailed studies were conducted on 45 patients attending the National Thalassemia Center who were chosen at random from more than 200 patients with HbE β thalassemia who were being followed at the Center. Clinical and hematologic data on this group of patients have been published previously, together with a detailed account of a classification system and hematologic data on this group of patients have been published previously, together with a detailed account of a classification system and the presence of red blood cells.

**Procedures**

Venous blood was collected into heparin and EDTA from all study participants. Duplicate measurements of methemoglobin levels and P50 were made from the heparinized blood sample with the use of a Rapidpoint 405 analyzer with an integral co-oximeter (Bayer). This instrument incorporates a polychromator that allows the simultaneous measurements across the various fractions of hemoglobin in the range of 473-671 nm. To confirm that methemoglobin levels were being measured accurately, blood samples from the propositus and a group of patients with HbE β thalassemia and normal controls were analyzed via the manual method of Evelyn and Malloy. We found there was close agreement between the methemoglobin values between the 2 methods used.

The samples were then centrifuged, the plasma removed, and plasma ascorbate levels measured immediately with a ferric-reducing ascorbate assay (procedure K671; BioVision). To prevent plasma protein precipitation, the ferric-reducing ascorbate buffer was diluted 1 in 10 before use. Plasma hemoglobin was measured with the use of a commercial assay (procedure TP801; Tridelva Development Ltd). Routine hematologic indices were measured in the EDTA sample (Coulter Electronics). The sample was centrifuged, the plasma removed from the cells, and both were stored at −20°C until shipped to Oxford on dry ice. Plasma erythropoietin and IL-8 levels were measured with an enzyme-linked immunosorbent assay kit (DEP00; R&D Systems) and a Compact human IL-8 ELISA kit (M1918; PeliKine). DNA was extracted from the cell pellet with the use of a QIAGEN DNA blood mini kit (51104), and the haplotype genotype was determined by polymerase chain reaction. Hemoglobin analysis, serum ferritin levels, and hepatic iron concentrations (measured by magnetic resonance imaging) followed previously reported methods.

To investigate the patient with a markedly increased methemoglobin concentration, further blood samples were collected from her and her immediate family and transferred into EDTA and acid citrate dextrose. EDTA samples were screened for glucose-6-phosphate dehydrogenase (G6PD) deficiency with the use of a qualitative assay (procedure 400; Trinity Biotech). Levels of reduced glutathione were determined (kit 371757; Calbiochem) and a red cell hemolysate, stabilized in EDTA-mercaptoethanol, was prepared and used for the measurement of cytochrome b5 reductase and glyceroldehyde phosphate dehydrogenase. A red cell hemolysate was prepared from each acid citrate dextrose sample, and G6PD activity was measured with a quantitative ultraviolet, kinetic assay (procedure 345-uv; Trinity Biotech Co). Glutathione reductase was measured using a quantitative manual method (kit GR2368; Randox Laboratories). Pyruvate kinase was measured according to the method described by Dacie and Lewis. Both cytochrome b5 and cytochrome b5 reductase genes and the HBA and HBB genes were sequenced.

A urine sample was collected from the patient and tested with Combur 10 diagnostic strips (Roche Diagnostics) for the presence of nitrites and hemoglobin. The urine sediment was examined by microscopy for the presence of red blood cells.

### Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (Release 16.0.1; SPSS Inc). Differences between median methemoglobin concentrations were assessed with the Mann-Whitney U test. We used multiple regression analysis to explore the relationship between methemoglobin and the potential predictor variables severity, transfusion status, and splenectomy. P < .05 was considered statistically significant.

### Ethical approval

Approval for the research program on HbE β thalassemia was obtained from the Ethical Committee of the College of Pediatricians, Colombo, Sri Lanka, and the Oxford Tropical Research Ethical Committee, Oxford, United Kingdom. This study was conducted in accordance with the Declaration of Helsinki.

### Results

The findings in the family that led to these studies are summarized in Table 1 and further biochemical data of the propositus, including findings before and after treatment, in Table 2. The propositus had a hemoglobin pattern typical of HbE β thalassemia; sequencing of the HBB genes revealed the β0 mutation on 1 chromosome and the severe β thalassemia mutation, IVS1-5 (G-C), which is very common in the Sri Lankan population, on the other.

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**Table 1. Hematologic and related studies in the propositus, before treatment with vitamin C, and her relatives**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>HbF (%)</th>
<th>HbA2 (%)</th>
<th>HbE (%)</th>
<th>Met Hb (%)</th>
<th>P50 (mm Hg)</th>
<th>CytoB5r (U/gHb)</th>
<th>GAPD (U/gHb)</th>
<th>Glutathione reductase (U/gHb)</th>
<th>G6PD deficiency screening test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>6.4</td>
<td>74.3</td>
<td>21.0</td>
<td>&lt; 1</td>
<td>2.3-3.2</td>
<td>76.7</td>
<td>10.7-13.6</td>
<td>21.3-23.6</td>
<td>50.7</td>
<td>301.5</td>
<td>9.81 Normal</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>10.7</td>
<td>54.9</td>
<td>15.8</td>
<td>&lt; 1</td>
<td>4.2</td>
<td>1.9</td>
<td>26.5</td>
<td>28.9</td>
<td>271.0</td>
<td>13.0 Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>14.2</td>
<td>78.0</td>
<td>23.9</td>
<td>&lt; 1</td>
<td>24.9</td>
<td>0.7</td>
<td>25.2</td>
<td>17.7</td>
<td>230.0</td>
<td>5.5 Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brother</td>
<td>14.6</td>
<td>81.5</td>
<td>28.5</td>
<td>&lt; 1</td>
<td>2.6</td>
<td>0.5</td>
<td>25.2</td>
<td>14.6</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sister</td>
<td>13.0</td>
<td>84.5</td>
<td>29.6</td>
<td>&lt; 1</td>
<td>2.6</td>
<td>0.4</td>
<td>25.3</td>
<td>21.8</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CytoB5r indicates cytochrome b5 reductase; GAPD, glyceroldehyde phosphate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; MCH, mean cell hemoglobin; Met, methemoglobin; and MCV, mean cell volume.
sequencing of the HBA and HBB genes revealed no other abnormalities, excluding hemoglobin M mutations. The mother had findings typical of β thalassemia trait, although complicated by iron deficiency anemia because of menorrhagia. The father had hemoglobin E trait, whereas the patient’s 2 siblings were healthy.

Multiple estimations indicated that the propositus had markedly increased levels of methemoglobin in the range of 10.7%-13.6%. The patient’s mother with β thalassemia trait had a slightly increased level of methemoglobin, whereas the levels in other family members were normal. The cytochrome b\(_5\) reductase levels increased level of methemoglobin, whereas the levels in other family members. Both cytochrome b\(_5\) (cytb\(_5\)) and cytochrome b\(_2\) reductase (cytb\(_5\)r) genes from the propositus were sequenced and showed no abnormality. Glyceraldehyde phosphate dehydrogenase and glutathione reductase levels were within the normal range in the propositus and both her parents. The patient’s P\(_{50}\) was significantly reduced compared with other family members, resulting in a marked left shift in the oxygen-dissociation curve compared with those of patients in the same population with HbEthalassemia trait, although complicated by iron deficiency anemia because of menorrhagia. Their division into strictly defined severity groups, as described in “Procedures,” main clinical and hematologic findings and some of the genetic modifiers responsible for the variation in their phenotypic severity have been reported previously. The major findings in these patients in relationship to the present study are summarized in Tables 3 and 4. As shown in Table 3, those with HbEβ thalassemia had a significant increase in methemoglobin compared with healthy controls and univariate analysis showed that there was a highly significant increase in methemoglobin levels in those who had been splenectomized compared with those who had intact spleens. As shown in Table 3, there was also a significant relationship between methemoglobin levels and phenotypic severity, as judged by the findings in the mild and severe groups and mirrored by the transfusion requirements. In multiple regression analysis, only splenectomy was statistically significantly related to methemoglobin level (standardized β = 0.64, t = 3.68, P < .01).

As shown in Table 4, the mean plasma ascorbate level in this group of patients was at the bottom limit of the normal range; in 10 cases it was subnormal. Although the number of cases available with matched plasma ascrobate and methemoglobin levels was too few to allow a statistical comparison, the levels were uniformly low.

### Table 2. Further biochemical and related analyses of the propositus, including, in some cases, data obtained before and after treatment with vitamin C

<table>
<thead>
<tr>
<th>Variable (units), normal range</th>
<th>Treatment period</th>
<th>Ascorbate (mmol/L), 28-84</th>
<th>Met Hb (%), 0.1-0.6</th>
<th>P(_{50}) (mm Hg), 24.1-27.2</th>
<th>Mean Hb (g/dL), 12-16</th>
<th>Mean Epo (IU/mL), 2-14</th>
<th>G6PD (U/gHb), 4.6-13.5</th>
<th>Pyruvate kinase (IU/gHb), 7.2-14.0</th>
<th>GSH, (μmol/gHb), 6.6-10.0</th>
<th>Haptoglobin (g/dL), 0.3-2.0</th>
<th>Hepatic iron (mg/g dw), 0.6-1.2</th>
<th>IL-8 (pg/mL), &lt; 10</th>
<th>Urinary nitrate, screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td></td>
<td>2.1-6.7</td>
<td>10.7-13.6</td>
<td>21.3-23.6</td>
<td>6.2</td>
<td>94.3</td>
<td>21.1</td>
<td>7.7</td>
<td>6.18</td>
<td>0.06</td>
<td>2.9</td>
<td>179.6</td>
<td>Negative</td>
</tr>
<tr>
<td>Posttreatment</td>
<td></td>
<td>30.2-36.6</td>
<td>0.85</td>
<td>26.9</td>
<td>7.0</td>
<td>76.8</td>
<td>6.18</td>
<td>0.26</td>
<td>2.9</td>
<td>179.6</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hb and Epo values are the means of 5 estimations.

Epo indicates erythropoietin; G6PD, glucose-6-phosphate dehydrogenase; Hb, hemoglobin; and Met, methemoglobin.

### Table 3. Analysis of methemoglobin levels in 45 patients with HbEβ thalassemia with a breakdown of cases into splenectomized and nonsplectomized, low and high transfusion rates, and mild and severe phenotypes as defined in the text

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Median Met Hb, %</th>
<th>Interquartile range</th>
<th>Range</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>17</td>
<td>0.3</td>
<td>0.25-0.4</td>
<td>0.1-0.6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>HbEβ thalassemia (all cases)</td>
<td>45</td>
<td>2.7</td>
<td>1.9-3.65</td>
<td>0.9-6.3</td>
<td></td>
</tr>
<tr>
<td>HbEβ thalassemia (splenectomized)</td>
<td>20</td>
<td>3.7</td>
<td>3.1-4.2</td>
<td>0.9-6.3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>HbEβ thalassemia (spleen intact)</td>
<td>25</td>
<td>2.3</td>
<td>1.5-2.8</td>
<td>0.9-4.8</td>
<td></td>
</tr>
<tr>
<td>HbEβ thalassemia (mild)</td>
<td>25</td>
<td>2.45</td>
<td>1.9-3.6</td>
<td>0.9-4.8</td>
<td>.084</td>
</tr>
<tr>
<td>HbEβ thalassemia (severe)</td>
<td>20</td>
<td>3.1</td>
<td>1.9-3.9</td>
<td>0.9-6.3</td>
<td></td>
</tr>
<tr>
<td>HbEβ thalassemia (0-20 blood transfusions)</td>
<td>21</td>
<td>2.6</td>
<td>1.85-3.1</td>
<td>0.9-3.6</td>
<td>.001</td>
</tr>
<tr>
<td>HbEβ thalassemia (&gt; 20 blood transfusions)</td>
<td>18</td>
<td>3.65</td>
<td>3.4-4.5</td>
<td>1.6-6.3</td>
<td></td>
</tr>
<tr>
<td>HbEβ thalassemia (mild, spleen intact)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
<td>.018</td>
</tr>
<tr>
<td>HbEβ thalassemia (mild, splenectomized)</td>
<td>12</td>
<td>3.6</td>
<td>2.65-4.05</td>
<td>0.9-5.0</td>
<td>.002</td>
</tr>
<tr>
<td>HbEβ thalassemia (severe, spleen intact)</td>
<td>12</td>
<td>2.65</td>
<td>1.38-3.18</td>
<td>0.9-4.8</td>
<td>.32</td>
</tr>
<tr>
<td>HbEβ thalassemia (severe, splenectomized)</td>
<td>8</td>
<td>4.0</td>
<td>3.7-4.45</td>
<td>3.5-6.3</td>
<td></td>
</tr>
<tr>
<td>HbEβ thalassemia (mild, spleen intact)</td>
<td>13</td>
<td>2.2</td>
<td>1.75-2.5</td>
<td>0.9-3.6</td>
<td>.01</td>
</tr>
<tr>
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<td>2.65</td>
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<td>3.5-6.3</td>
<td></td>
</tr>
</tbody>
</table>

Hb, hemoglobin; and Met, methemoglobin.
small to determine whether there was a significant relationship between the 2 at this level of plasma ascorbate, the mean level of methemoglobin in those in whom these measurements were available and who had all been splenectomized was 4.24%, and the mean level of plasma ascorbate was 23.1 nmol/mL, that is in the subnormal range.

Overall, the level of haptoglobin was subnormal in the patients with HbE β thalassemia, although no case of absent haptoglobin was encountered. The majority of the patients had the 2.2 haptoglobin genotype. There was a wide range of hepatic iron concentrations that were not significantly related to the methemoglobin level. There was a highly significant elevation of IL-8 levels in this group in these patients requires further study. The most striking finding, however, was the highly significant relationship between ascorbate and hypoxia response in humans and mice are similar.

What are the broader issues resulting from these findings? In particular, because the results of the studies in this unusual patient provide clear evidence that ascorbate deficiency can induce methemoglobinemia in HbE β thalassemia, how common are increased methemoglobin levels in this condition and are the levels related mainly to ascorbate or are other factors involved? There have been relatively few reports of the levels of methemoglobin in the inherited hemoglobin disorders. An early study of a few cases of HbE β thalassemia in northern India suggested that methemoglobin levels might be increased in this condition,26 and increased levels have been reported in some cases of inherited unstable hemoglobins27 and sickle cell anemia.28,29

In the present study, there was a significant increase of methemoglobin in a group of patients with HbE β thalassemia whose mean level of plasma ascorbate was at the lower limit of normal; 10 cases showed subnormal levels. However, no cases were encountered with a reduction to the level found in the propositus in this study and the extent to which ascorbate deficiency may be responsible for the modest increase in methemoglobin in these patients requires further study. The most striking finding, however, was the highly significant relationship between splenectomy and methemoglobin levels together with the effect of phenotypic severity, including blood transfusion status. Because the main factors underlying phenotypic variability in this group of patients identified so far are the coinheritance of α thalassemia or relatively high levels of HbE,30 both of which modify the
degree of globin-chain imbalance, it seems likely that splenic function and the degree of excess α-chain synthesis play a major role in determining the level of methemoglobin, at least in HbE β-thalassemia.

What is the source of the increased methemoglobin? As in other forms of β-thalassemia, excess α-chains are produced in HbE thalassemia with the production of red cell inclusions;31 despite the mild instability of HbE β chains are not found in these precipitates.32 One of the major degradation products of excess α-chains are hemichromes, which bind to the red cell membrane and promote sequestering of band 3.33 As they form, they go through reversible and irreversible phases during which methemoglobin is produced as an intermediate. It is possible, therefore, that abnormal red cells exposed to this mechanism are recognized and sequestered in the spleen and hence the level of methemoglobin is increased after splenectomy. Because, like other forms of thalassemia, there is a significant hemolytic component in HbE β-thalassemia, it follows that the circulation will be continuously exposed to increased levels of methemoglobin.

Another potential source of methemoglobin, in this case in our case, is the further oxidation of hemoglobin released during hemolysis, the fail-safe mechanism in this case again is binding by haptoglobin. In the present study the haptoglobin levels were reduced in the patients with HbE β-thalassemia, although only to a minor degree. However, molecular analysis showed that in almost every case the haptoglobins were of the 2.2 variety, which has been shown to be less effective than the 1.1 variety with respect to hemoglobin binding and which occurs commonly in some Asian countries.34 Recent studies suggest that of its reduced binding properties, it may put greater pressure on the use of ascorbate or as a free radical scavenger and, indeed, may be associated with increased frequency of the clinical manifestations of ascorbate deficiency.11,12,35

Methemoglobin is a significant activator of endothelial cells by stimulation of E-selectin, IL-6, and IL-8 production.10 It is of interest therefore that the IL-8 levels in this series of patients with HbE β-thalassemia were considerably increased. Because of increasing evidence for vascular complications in other forms of thalassemia intermedia36 and in sickle cell disease, and because of the results of the small pilot study shown in Table 5, further investigation of the potential pathologic role of methemoglobin is indicated, particularly in conditions with reduced splenic function or in which splenectomy is commonly practiced.

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Authorship

Contribution: A.A. and C.F. performed the laboratory studies; S.A. conducted the statistical analysis; A. Premawardhena, D.B., A. Perera, T.S.P., and N.O. collected and analyzed the clinical data on the patients, and A.A. and D.J.W. designed the study and wrote the manuscript.
Conflict-of-interest disclosure: The authors declare no competing financial interests.
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