Interaction of Hemoglobin E and Pyrimidine 5’ Nucleotidase Deficiency

By David C. Rees, John Duley, H.A. Simmonds, B. Wonke, S.L. Thein, J.B. Clegg, and D.J. Weatherall

A Bangladeshi family is described in which the genes for both hemoglobin E (Hb E) and pyrimidine 5’ nucleotidase deficiency are segregating. An individual homozygous for both these conditions has a severe hemolytic anemia, whereas family members who are homozygous for Hb E are asymptomatic and those homozygous for pyrimidine 5’ nucleotidase deficiency have the mild hemolytic anemia that is characteristic of this disorder. Globin-chain synthesis experiments have shown that the mechanism underlying the interaction between these two genotypes is a marked decrease in the stability of Hb E in pyrimidine 5’ nucleotidase-deficient red blood cells (RBCs). It has also been found that in the enzyme-deficient RBCs in which Hb E is highly unstable, free α-chains, though not βE-chains, accumulate on the membrane. In view of the increasing evidence that the hemolysis associated with pyrimidine 5’ nucleotidase deficiency results not only from an increase in the level of erythrocyte pyrimidines, but also from inhibition of the hexose monophosphate shunt activity in young erythrocytes, it is likely that the marked instability of Hb E in the enzyme-deficient cells results from oxidant damage acting on a mildly unstable Hb variant. These observations may have important implications for the better understanding of the pathophysiology of Hb E/β-thalassemia, globally the commonest important form of thalassemia.

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MATeRIALs AND METHODS

Case history. The propositus presented at age 7 years with pallor, fever, and malaise. She was the youngest of five children born to Bangladeshi parents, who were first cousins. She was pale and jaundiced, with the facial changes associated with marrow expansion, and on the 25th centile for height and weight. She had hepatosplenomegaly with the liver edge 5 cm and the spleen 10 cm below the costal margin. Her 18-year-old sister also suffered from an undiagnosed hemolytic anemia. Hb level 7 to 8gL: she had developed normally, following the 50th centile for height and weight, with no symptoms of anemia, normal facial appearance, and no hepatosplenomegaly. The parents and three brothers were all well. The family tree is shown in Fig 1.

The propositus was given a series of blood transfusions before splenectomy. Postsplenectomy, she was able to maintain an Hb level of 7 to 8gL, becoming transfusion independent. Her weight has risen to the 75th centile, her height to the 50th centile. Her facial appearance and development have become normal over the last 4 years.

Hematologic analysis. Blood was collected into EDTA. Full blood counts were performed using an automated counter. Blood films, reticulocyte stains, Hb electrophoresis, isopropanol stability tests, and acidified glycerol lysis tests were performed by standard techniques.12,13

DNA analysis. DNA was isolated from peripheral blood leukocytes.11 The β-globin genes of the anemic individuals were sequenced, after polymerase chain reaction (PCR)-amplification with previously published conditions and primers.15 One of the PCR primers in the reaction was 5’-biotinylated, and a single-stranded template isolated using magnetic beads (Dynal M-280 Streapavidin) and a magnetic particle concentrator (MPC-E; Dynal, Wirral, UK).16

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The 5’ and 3’ β-globin haplotypes of the family were determined using PCR-based techniques.

Family members were screened for α-thalassemia by digesting DNA with BglII restriction enzyme, Southern blotting, and hybridization with a radio-labeled ζ-specific probe.

**RBC enzyme assays and nucleotide profiles.** The following glycolytic enzymes were assayed in the anemic individuals (II.4 and II.5) using standard techniques: G6PD (glucose-6-phosphate dehydrogenase), PK (pyruvate kinase), glucose phosphate isomerase, glutathione reductase, and 6-phosphogluconate dehydrogenase.

The isoenzyme of pyrimidine 5’ nucleotidase whose deficiency causes hemolysis, uridine monophosphate hydrolyase-1 (UMPH-1), was assayed using a high-performance liquid chromatography (HPLC)-based method which measures the rate of breakdown of uridine monophosphate to produce uridine. UMPH-2 activity was measured as a control, using the breakdown of deoxy-uridine monophosphate. The erythrocyte nucleotides and their conjugates were measured by acid precipitation and anion-exchange HPLC.

**Globin-chain biosynthesis.** Heparinized venous blood was kept on ice until incubated at 37°C with tritiated leucine in a plasma-based incubation medium, within 2 hours of venesection. White blood cells were removed before incubation using a-cellulose and microcrystalline cellulose (SigmaCell 50; Sigma Chemical Co, Poole, Dorset, UK).

For each family member the incubation was continued for 1 hour before washing the cells with cold reticulocyte saline (0.13 mol/L NaCl, 0.005 mol/L KCl, 0.007 mol/L MgCl2), followed by acid-acetone precipitation of the globin from whole cells. The globin chains were separated by eation exchange chromatography and the radioactivity into each fraction measured by scintillation counting. A linear two-chamber gradient, producing 120 fractions, was used for separating Hb E samples (I.1, I.2, I.3, I.4, and II.5). In the case of family members II.4 and II.5, a time-course incubation was also performed, with aliquots removed at 15, 60, and 120 minutes. Globin was prepared as before and the chains separated. Specific activities of the chains were measured at each time, after dialysis of the peak fractions against 0.5% formic acid. After 60 minutes, an aliquot was removed, and after washing, lysed with 20 mOsm phosphate buffer pH 7.4. The lysate was ultracentrifuged at 80,000g for 10 minutes. The supernatant was removed, converted to globin, the globin chains separated, and the radioactivity of the fractions measured by scintillation counting, as for the whole cell lysate. The membrane ghosts were washed four times using the phosphate lysing buffer, before being resuspended with 80 mg of unlabeled “carrier” Hb and made into globin. This is a modification of Dodge’s method to produce Hb-free ghosts. This procedure was also followed on an Hb E homozygote as a control (not a member of this family).

**RESULTS**

**Hematologic analysis.** Relevant data are summarized in Table 1. On admission, the propositus (II.5) was severely anemic, with an Hb level of 2.8g/dL, mean cell volume (MCV) 59.7 fl, reticulocyte count $0.12 \times 10^5$/L. A blood film showed marked anisopoikilocytosis with prominent basophilic stippling; no nucleated RBCs or Heinz bodies were seen. Hb electrophoresis showed one band only, corresponding to Hb E/A2. Her anemic sister (II.4) had an Hb value of 7.7 g/dL, MCV 90.2 fl, and a reticulocyte count $0.30 \times 10^5$/L; a blood film showed mild anisopoikilocytosis and prominent basophilic stippling. Hb electrophoresis showed Hb A and 2.5% Hb A2 only. Both parents and one of the brothers (II.3) were found to be carriers of Hb E; one brother (II.2) was homozygous for Hb E; all of these had the expected phenotypes. The third brother (II.1) had never had a blood test and only a small volume of blood was available for analysis. He was mildly anemic, with basophilic stippling.

The isopropanol stability test showed mild cloudiness after 20 minutes in II.2, II.3, and II.5, consistent with mild instability of Hb E. There was no cloudiness after 30 minutes in II.4, making the presence of an unstable Hb variant unlikely.

The results of the acidified glycerol lysis test on II.4 and II.5 are shown in Fig 2: II.5 has an increased resistance to hemolysis, consistent with the thalassemic phenotype of Hb E; there is no evidence of a membranopathy contributing to the hemolysis.

**DNA analysis.** Sequencing of the β-globin genes of the propositus showed homozygosity for codon 26 GAG → AAG (Hb E); her sister (II.4) showed no abnormality. Southern blotting on all the family members (except II.1) showed no evidence of deletional α*-thalassemia. The results of β-globin haplotyping are summarized in Table 2. They confirm that hemolysis (II.4 and II.5) does not segregate with the β-globin complex.

**RBC enzyme assays and nucleotide profiles.** The glycolytic enzymes of the anemic sisters were all within the expected limits (Table 3). However, UMPH-1 activity was markedly reduced in both sisters, suggesting the diagnosis of pyrimidine 5’ nucleotidase deficiency; it was equally reduced in II.1, who had not previously had a blood test. Other family members all showed activities consistent with carrier status for PS’N deficiency (Table 3). UMPH-2 levels, a control measurement, were normal in all cases.

There was also a massive accumulation of the pyrimidine
HEMOGLOBIN E AND 5'N DEFICIENCY

Table 1. Summary of Hematologic Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>RBC (x10^12/L)</th>
<th>Reticulocytes (%)</th>
<th>% Hb E + A2</th>
<th>α/Non-α Total Counts' Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1</td>
<td>11.7</td>
<td>65.7</td>
<td>5.55</td>
<td>0.5</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>I.2</td>
<td>13.2</td>
<td>71.3</td>
<td>5.71</td>
<td>0.5</td>
<td>26</td>
<td>1.5</td>
</tr>
<tr>
<td>II.1</td>
<td>11.2</td>
<td>91</td>
<td>3.92</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>II.2</td>
<td>13.6</td>
<td>60.8</td>
<td>7.04</td>
<td>1</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>II.3</td>
<td>14.8</td>
<td>70.7</td>
<td>6.47</td>
<td>0.5</td>
<td>30</td>
<td>1.5</td>
</tr>
<tr>
<td>II.4</td>
<td>7.6</td>
<td>90</td>
<td>2.95</td>
<td>7</td>
<td>2.6</td>
<td>0.9</td>
</tr>
<tr>
<td>II.5 (presplenectomy)</td>
<td>2.8</td>
<td>59.7</td>
<td>1.71</td>
<td>7</td>
<td>97</td>
<td>*</td>
</tr>
<tr>
<td>II.5 (postsplenectomy)</td>
<td>8.3</td>
<td>82</td>
<td>3.35</td>
<td>40</td>
<td>94</td>
<td>2</td>
</tr>
</tbody>
</table>

* Insufficient blood for analysis.

nucleotides uridine triphosphate and cytidine triphosphate in the UMFP-1-deficient individuals (579 and 882 mmol/L erythrocytes, respectively, in II.4; 1,224 and 3,167 in II.5); these are normally undetectable (<1 mmol/L erythrocytes). In addition, there were very elevated concentrations of uridine diphosphate glucose and cytidine nucleotide conjugates of lipids. These findings are consistent with the diagnosis of pyrimidine 5' nucleotidase deficiency.

Globin-chain biosynthesis. The 1-hour total α/β synthesis ratios for the family members are given in Table 1. In the Hb E heterozygotes (I.2, I.3), apart from the mother (I.1), the α/β synthesis ratios are in the expected range of 1.5. The balanced synthesis in the mother may reflect the presence of nondeletional α-thalassemia, although one might have expected a lower percentage of Hb E in her blood if this were the case. Both Hb E homozygotes (II.2, II.5) show greater chain imbalance, with α/β ratios of 2.0.

The time-course incubations on II.4 and II.5 are shown in Figs 3 and 4. The increase in specific activity is linear for the less severely affected sister (II.4, Hb A), as is expected for synthesis of a stable Hb molecule. However, the increase of specific activity in the propositus (II.5, Hb E) plateaued after 1 hour, with little increase during the second hour. This is the pattern seen in unstable Hbs, such as Hb Bristol, the plateau occurring when the newly synthesized chains start to precipitate and undergo proteolysis.26 The fact that this effect occurs in both α- and β-chains suggests that the whole Hb molecule is unstable, as has been shown for classical unstable Hbs.26,27 A control time-course using homozygous Hb E showed linear synthesis over 2 hours (Fig 5).

The results of globin-chain separation of the stringently washed membranes, after 1 hour's incubation, are shown in Figs 6 and 7. II.4 showed significant amounts of both α- and β-globin chains bound to the membrane, in the ratio α/β 0.7. Although this could represent a failure to wash away all the unbound globin, the change in the bound α to β ratio compared with the whole-cell lysate (α/β 0.9) supports the possibility that these chains are differentially covalently bound to the membrane. The membrane separation from II.5 showed a large excess of a chains, with a small peak in the pre-αβ region. This pattern is similar to that found in β-thalassemia major.28 The control homozygous Hb E sample showed no detectable globin bound to the membrane.

DISCUSSION

Considered separately, neither Hb E nor pyrimidine 5' nucleotidase deficiency can explain the severity of the anemia of the propositus. This is clearly shown by the phenotypes of her siblings, two with pyrimidine 5' nucleotidase deficiency without Hb E (II.1, II.4) and one with homozygous Hb E (II.2) (Table 1). There is no evidence of any other trait, such as a membranopathy, segregating within this family, and so it is very likely that it is the interaction between the Hb E and pyrimidine 5' nucleotidase deficiency which produces the marked anemia in the propositus.

The nature of this interaction is clarified by the time-course globin synthesis experiments. The nonlinear increase in specific activity over 2 hours in the propositus is typical of rapid precipitation and proteolysis of the newly synthesized Hb, as occurs in unstable Hbs (Figs 6 and 7).26 The alternative explanation is that the rate of synthesis of globin chains in the propositus decreases during the 2 hours of the incubation: this would imply that the reticulocytes of the proband are metabolically vulnerable, but the very high rate of radioactive incorporation during the incubation argues against this: for example, the specific activity of the α-chains...
in the propositus (II.5) is more than four times higher than that in her sister (II.4).

The mechanism of premature RBC destruction in patients with pyrimidine 5' nucleotidase deficiency is not absolutely clear. Although earlier studies suggested that it might be the result of abnormal accumulation of pyrimidines within the RBC, more recent work provides clear evidence that, in addition, the accumulation of nucleotides has a marked inhibitory effect on the pentose phosphate shunt. These observations, which are in keeping with earlier studies which showed an increase in Heinz body formation after incubating pyrimidine 5' nucleotidase-deficient RBCs with acetylphenylhydrazine, suggest that in addition to pyrimidine accumulation these cells may be sensitive to oxidant stress and that their Hb may undergo oxidant damage. In this context, it is interesting that the RBC membranes of the homozygote for pyrimidine 5' nucleotidase deficiency who did not have Hb E contained small but significant quantities of both α- and β-globin chains. In the enzyme deficient homozygote who was also homozygous for Hb E there was a significant excess of free α-chains bound to the membrane, although no β'-chains could be demonstrated. We have obtained similar findings in patients with Hb E/β-thalassemia (unpublished observation, 1995). This suggests that if Hb E does indeed precipitate in metabolically stressed RBCs it does not form aggregates that become attached to the RBC membrane, like free α-chains or unstable Hbs.

The metabolic stress within the RBCs caused by the pyrimidine 5' nucleotidase deficiency could act in two ways: the hostile intracellular environment may exacerbate the instability of Hb E such that the molecule precipitates and generates oxidative stress within the RBC in a way similar to typical unstable variants; secondly, the proteolytic capacity of the RBCs to cope with the excess α-chains, resulting from the mildly thalassemic nature of the βE-allele, may be reduced by the pyrimidine 5' nucleotidase deficiency, creating a phenotype similar to severe thalassemia. It has recently been suggested that the oxidative damage caused by the excess free α-chains in β-thalassemia is greatly potentiated by the presence of reduced glutathione; this mechanism may be important in the pathogenesis of the anemia in the propositus, in that free α-chains associated with the Hb E could interact with excess reduced glutathione known to be present in pyrimidine 5' nucleotidase-deficient RBCs.

The clinical picture in the propositus more closely resembles that of an unstable Hb, supporting the first mechanism: presplenectomy there was a significant reticulocytosis that increased dramatically postsplenectomy; equally the α/ non-α globin chain synthesis ratio of 2.0 is identical to that of the brother (II.2) with homozygous Hb E, which does not suggest a worsening of the chain imbalance. Similarly, there is not a marked increase in level of Hb F, suggesting that hemolysis predominates over ineffective erythropoiesis. In favor of a thalassemic-like mechanism is the microcytosis (presplenectomy) and the excess α-chains binding to the membrane. Both mechanisms probably play a part, but instability and hemolysis seem to predominate over ineffective erythropoiesis. Interestingly, the response to splenectomy was very good, leading to an increase in Hb level, an increase in growth rate, and a reversal of the bony changes. This clinical response to splenectomy is again not typical of Hb E/β-thalassemia, nor pyrimidine 5' nucleotidase deficiency, but more similar to that in patients with unstable Hbs.

There is very little previous evidence that the instability

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Table 2. β-Globin Haplotypes on Family Members

<table>
<thead>
<tr>
<th>Epsilon/HindII</th>
<th>G Gamma/HindIII</th>
<th>A Gamma/HindIII</th>
<th>Pseudobeta/HindII</th>
<th>3' Pseudobeta/HindII</th>
<th>3'/Hpal</th>
<th>3'/HindII</th>
<th>3'/BamHI</th>
<th>Hb Status</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1 +/+</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
<td>AE</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.2 +/+</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/-</td>
<td>AE</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.3 *</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.4 +/+</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/+</td>
<td>AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.5 +/+</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>EE</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hb A is linked to the +++++ haplotype and Hb E to +++++. Hemolysis does not segregate with Hb E or the β-globin haplotype.

* Units are nmol/hg

Table 3. Results of Assays for RBC Enzymes

<table>
<thead>
<tr>
<th>G6PD (IU)</th>
<th>6PGD (IU)</th>
<th>PK (IU)</th>
<th>GPI (IU)</th>
<th>GSHRx (IU)</th>
<th>UMPH-1*</th>
<th>UMPH-2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>I.2</td>
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<td>3</td>
<td>7</td>
</tr>
<tr>
<td>I.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>I.4</td>
<td>1.34</td>
<td>1.33</td>
<td>4.9</td>
<td>17.1</td>
<td>4.6</td>
<td>0.1</td>
</tr>
<tr>
<td>I.5</td>
<td>3.35</td>
<td>1.69</td>
<td>10.4</td>
<td>19.2</td>
<td>7.2</td>
<td>0.5</td>
</tr>
<tr>
<td>NR</td>
<td>1.2-1.72</td>
<td>1.0-1.42</td>
<td>2.0-4.0</td>
<td>12.3-27.9</td>
<td>2.0-10.8</td>
<td>9-20</td>
</tr>
</tbody>
</table>

Normal ranges are shown in the bottom row.

* Units are nmol/mg Hb/h.
Hemoglobin E and P5'N Deficiency

of Hb E is important in vivo. Dapsone is reported to have caused hemolysis in a Cambodian woman with Hb E trait. Unfortunately, it was not possible to exclude G6PD deficiency in the steady state in this case, and it would be expected that such a reaction would have been noticed more widely, given the large numbers of Hb E carriers who must have been exposed to dapsone. A second study indirectly implicated instability of Hb E by noting that, while neither homozygous nor heterozygous states for Hb E offered protection against falciparum malaria, some protection occurred in these groups if they had consumed fava beans in the preceding month; again this study has not been duplicated.

Surprisingly, there has been no convincing study looking at the interaction of Hb E and G6PD deficiency, although they must commonly occur together. The coincidence of these two traits has been noted, but with no apparent alteration of phenotype, although this was not specifically studied. There are no studies of hemolytic crises in people with Hb E and G6PD deficiency to see if they are more severely affected than those with Hb A or if hemolysis preferentially destroys Hb E. Similarly, there are no studies of Hb E associated with other RBC enzyme deficiencies. There are reports of Hb E occurring with hereditary ovalocytosis and hereditary elliptocytosis, with no increase in hemolysis.

It is interesting that all members of our family appear to have partial or complete pyrimidine 5' nucleotidase deficiency. Although this may reflect the segregation of the pyrimidine 5' nucleotidase deficient trait through the family, it has also been noted that thalassemia trait inhibits pyrimidine 5' nucleotidase activity; therefore, either of the brothers II.2 and II.3 may have an acquired reduction in pyrimidine 5' nucleotidase activity rather than true carrier status.

Homozygous Hb E and pyrimidine 5' nucleotidase deficiency therefore interact to produce a hemolytic anemia, significantly worse than that produced by either condition alone. A possible basis for this interaction is that the intracellular oxidative stress produced by the enzyme deficiency acts on the inherent instability of the Hb E and causes its premature destruction within the RBC. This is the first demonstration that the instability of Hb E can be significant in vivo, and may reflect an important mechanism in the pathophysiology of Hb E/β-thalassemia, another situation in which Hb E is exposed to unusual oxidant stress. In this condition the greater chain imbalance caused by the β-thalassemia allele, with the precipitation of free α-chains and damage to the RBC membrane would be the major cause of oxidant stress; this might lead to premature destruction of

Fig 3. Specific activity time-course over 2 hours for II.4. The specific activity increases in an approximately linear fashion, consistent with a constant rate of synthesis and stable Hb. (○), SA α; (●), SA β.

Fig 4. Specific activity time-course over 2 hours for II.5. The plateau in specific activity after 1 hour suggests instability of the newly synthesized Hb E molecules. The higher specific activity of the α-chains results from proteolysis of the excess, free α-chains resulting from the decreased rate of synthesis of the β'-globin. (●), SA β E; (○), SA α.

Fig 5. Specific activity time-course over 2 hours for a control with Hb E (but no other RBC abnormalities). The specific activity increases in a linear fashion. (●), SA α E; (○), SA α.
Fig 6. Globin chains bound to the membrane in 11.4 after stringent washing. The open circles/solid line denote radioactive incorporation, the solid circles/dotted line trace the optical density at 280 nm; this latter is predominantly the profile of the carrier globin added to help precipitate the membrane-bound globin. A two-chambered linear gradient was used during the cation-exchange chromatography. The ratio of α:β-chains is 0.7.

Fig 7. Globin chains bound to the membrane in 11.5 (A) and control Hb E homozygote (B). The open circles/solid line denote radioactive incorporation, the solid circles/dotted line trace the optical density at 280 nm; this latter is predominantly the profile of the carrier globin added to help precipitate the membrane-bound globin. A three-chambered convex gradient was used during the cation-exchange chromatography. A large peak of α-chains is seen with 11.5 and no detectable bound globin for the control sample.
Hb E, a mechanism that could contribute to the remarkable severity of this interaction.

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