Severe Hemolytic Anemia Associated With the Homozygous State for an Unstable Hemoglobin Variant (Hb Bushwick)

By P. Srivastava, J.S. Kaeda, D. Roper, T.J. Vulliamy, M. Buckley, and L. Luzzatto

We have investigated a 13-year-old girl from first cousin parents who presented with severe hemolytic anemia. Hematologic studies showed unstable hemoglobin (Hb) disease (chronic Heinz body anemia), and DNA analysis showed that the patient was homozygous for the previously reported abnormal Hb called Hb Bushwick (ββ′E18 Gly → Val). Hb Bushwick is unstable in vitro and in vivo. In addition, using globin chain biosynthetic studies, we show that the ββ′αα′ chains are unstable. Six members of the patient’s family were heterozygous for Hb Bushwick and had a compensated hemolytic disorder. By contrast, the homozygous patient had chronic anemia caused by a combination of hemolysis and ineffective erythropoiesis that was subject to severe exacerbation concomitant with infection. Thus, although unstable Hb disease is correctly regarded as dominant, we clearly see a dosage effect in its expression, whereby the homozygous state is still compatible with life although the red blood cells contain nearly 100% unstable Hb.

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Abnormally unstable hemoglobins (Hbs) are a well-recognized cause of chronic hemolytic anemia, and some 90 Hb variants causing this type of pathology have been reported. Because Hb precipitates can be visualized with supravital stains as Heinz bodies, unstable Hb disease is also referred to as chronic Heinz body anemia (CHBA). The main mechanism of hemolysis in this condition is probably the intracellular precipitation of the abnormal Hb, which renders the erythrocyte susceptible to removal by macrophages in the spleen and in other organs. In addition, at least in some cases there is evidence of intravascular hemolysis and ineffective erythropoiesis. Because low solubility is, by definition, an intrinsic property of unstable Hbs, precipitates form even in the presence of normal Hb, thus providing a good explanation for the dominant pattern of inheritance of CHBA. Indeed, all of the patients reported thus far have been heterozygotes.

We report here the first example of the homozygous state for an unstable Hb variant. This finding has given us the unusual opportunity to study erythrocytes containing Hb Bushwick (ββ′E18 Gly to Val) in a virtually pure state.

CASE REPORT

A 13-year-old Pakistani girl, the offspring of a marriage between first cousins, presented as an emergency case with an upper respiratory infection and severe anemia (Hb level, 3.8 g/dL). Because of impending heart failure she received a transfusion of 3 U of blood over a period of 48 hours. The patient had never received any blood transfusions previously. She was subsequently investigated and found to have persistent anemia (Fig 1), with reticulocytosis (Table 1), mild jaundice (bilirubin level, 25 μmol/L), and a spleen palpable to 5 cm below the costal margin. Iron studies (serum iron, total iron binding capacity [TIBC], and iron transferrin saturation), B12, and serum folate levels were all normal. The ferritin level was 32 and 26 μg/L on two occasions. The patient has not required any blood transfusions since the clinical episode that led to her initial investigation.

MATERIALS AND METHODS

Full blood counts were determined on a Sysmex 5000 (TOA-Medical Electronic Co Ltd, Kobe, Japan) and hematologic studies were performed using standard techniques. Hb electrophoresis was performed on cellulose acetate (pH 8.5) and citrate agar gel (pH 6.0). Hb F was quantitated by alkali denaturation and Hb A2 was estimated by elution from cellulose acetate electrophoretic strips. The presence of unstable Hb was investigated by both the isopropyl alcohol and the heat stability tests. To obtain a semiquantitative estimate of the unstable Hb, hemolysates were incubated at 50°C for 5 hours and at intervals of time the Hb remaining in solution was measured with Drabkin’s reagent at 540 nm. The O2 dissociation curve was performed and the 2,3-diphosphoglycerate (2,3 DPG) concentration was determined. Reticulocytes in whole blood from the patient’s sample were incubated at 37°C for 5 to 90 minutes with 3H-leucine in Krebs solution, and the biosynthetic globin chain ratio was determined after incubation times of 5, 20, 30, and 90 minutes.

A region of the β-globin gene was amplified using polymerase chain reaction (PCR), including exons 1 and 2 and intron 1, using genomic DNA as template and primers sitied at −158 to −138 (5′-AAGCCAGTGCCAGAAGAGCC-3′) and IVS 2 nt 4-17 (5′-ACA
tCAAGGGTCCATAGAC-3′). The amplified fragment was sequenced directly with internal oligonucleotides using the dideoxy chain termination method of Sanger et al following the sequencing version 2.0 protocol (USB-Amersham, UK) with the following modifications. The annealing mix was boiled for 10 minutes and snap frozen. The labeling reaction was incubated for 1 minute at room temperature. To rule out the coexistence of other gross abnormalities of the globin genes, both the α and β globin gene clusters were analyzed by Southern blot analysis, using genomic α, β, γ globin gene-specific probes.

RESULTS

Hematologic findings and family study. In the steady state, the patient had a mild to moderate normocytic, normochromic anemia, with evidence of chronic hemolysis (Table 1). The blood smear stained with May-Grüntwald Giemsa showed hypochromia with prominent punctate basophilia and some poikilocytosis (Fig 2A). Supravital stain showed...
Table 1. Hematology Data in Members of Family With Hb Bushwick

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)/Sex</th>
<th>Hb (g/dL)</th>
<th>RBC (10^12/L)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
<th>HbA2 (%)</th>
<th>HbF (%)</th>
<th>Retics (x10^6/L)</th>
<th>Heinz Bodies</th>
<th>β-Globin Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>66/M</td>
<td>14.3</td>
<td>4.78</td>
<td>93</td>
<td>29.9</td>
<td>32.3</td>
<td>4.1</td>
<td>0.8</td>
<td>110</td>
<td>+</td>
<td>ββ*</td>
</tr>
<tr>
<td>II-1</td>
<td>40/M</td>
<td>15.5</td>
<td>5.36</td>
<td>91</td>
<td>28.9</td>
<td>31.8</td>
<td>4.4</td>
<td>0.5</td>
<td>236</td>
<td>+</td>
<td>ββ*</td>
</tr>
<tr>
<td>II-2</td>
<td>37/F</td>
<td>13.0</td>
<td>4.31</td>
<td>91</td>
<td>30.2</td>
<td>32.9</td>
<td>4.4</td>
<td>1.8</td>
<td>215</td>
<td>+</td>
<td>ββ*</td>
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<tr>
<td>II-3</td>
<td>36/M</td>
<td>16.3</td>
<td>5.75</td>
<td>91</td>
<td>28.3</td>
<td>35.0</td>
<td>3.0</td>
<td>0.5</td>
<td>74</td>
<td>-</td>
<td>ββ*</td>
</tr>
<tr>
<td>II-4</td>
<td>34/F</td>
<td>11.8</td>
<td>4.14</td>
<td>94</td>
<td>28.6</td>
<td>30.3</td>
<td>4.0</td>
<td>0.7</td>
<td>207</td>
<td>+</td>
<td>ββ*</td>
</tr>
<tr>
<td>III-1</td>
<td>18/M</td>
<td>14.0</td>
<td>4.84</td>
<td>91</td>
<td>28.9</td>
<td>31.8</td>
<td>4.6</td>
<td>1.6</td>
<td>295</td>
<td>+</td>
<td>ββ*</td>
</tr>
<tr>
<td>III-2</td>
<td>13/F</td>
<td>10.1</td>
<td>1.53</td>
<td>95</td>
<td>28.6</td>
<td>30.0</td>
<td>5.0</td>
<td>1.7</td>
<td>578</td>
<td>++</td>
<td>ββ*, βB, βE</td>
</tr>
<tr>
<td>III-3</td>
<td>12/M</td>
<td>15.2</td>
<td>5.36</td>
<td>80</td>
<td>28.4</td>
<td>35.5</td>
<td>2.9</td>
<td>2.0</td>
<td>80</td>
<td>-</td>
<td>ββ*</td>
</tr>
<tr>
<td>III-4</td>
<td>8/F</td>
<td>10.5</td>
<td>3.72</td>
<td>86</td>
<td>28.4</td>
<td>32.8</td>
<td>4.6</td>
<td>2.2</td>
<td>75</td>
<td>+</td>
<td>ββ*</td>
</tr>
</tbody>
</table>

The homozygous patient is shown in bold type (III-2).

Abbreviations: MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; Retics, reticulocytes; ββ*, βBushwick.

* Increased levels are probably artefactual (see text).

Table 2. Globin Chain Biosynthesis in Patient Homozygous for Hb Bushwick

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Ratio βα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.0 (±0.05)</td>
</tr>
<tr>
<td>Mother (II-2)</td>
<td>0.88</td>
</tr>
<tr>
<td>Patient (III-3)</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.64</td>
</tr>
</tbody>
</table>

The smear on the electrophoretic strip suggested an unstable variant (Fig 3). Apart from the patient’s sister (III-3), had normal or nearly normal Hb levels, normal RBC indices, and minimal morphologic abnormalities (Fig 2B). However, six of the eight members of the family were able to test (all except II-2 and III-3) had significant reticulocytosis. In all of these, Heinz bodies could be readily induced by incubating a peripheral blood sample at 50°C for 1 hour. Both glucose-6-phosphate dehydrogenase (G6PD) and 2,3-DPG levels were increased to an extent consistent with reticulocytosis.

Hb studies. Hb electrophoresis on cellulose acetate (pH 8.5) of the patient’s fresh hemolysate yielded a normal pattern. However, after the hemolysate had been stored for 24 hours at 4°C, the pattern was modified in that a smear appeared in the region between Hb A and Hb A2 (Fig 4). Results of the family were obtained in the other family members with reticulocytosis. The apparent increase in Hb A2, ranging from 4.0% to 5.0% (normal range, 1.5% to 3.0%), must therefore be regarded as artifactual. Hb F levels were slightly increased in some but not all subjects who had the abnormal Hb (Table 1).

The smear on the electrophoretic strip suggested an unstable Hb, and this was confirmed by the demonstration of a heat-labile Hb component (Fig 5). After 5 hours at 50°C, 50% of the patient’s Hb was denatured (at longer incubation times normal Hb also begins to denature). Family members with a positive result of a heat stability test had estimated levels of 12% to 20% (control level, 2%). We inferred that the patient was homozygous for the unstable variant and that subjects I-1, II-1, II-2, II-4, III-1, and III-4 were heterozygous. Hemozygosis of the patient was compatible with both of her (consanguineous) parents being heterozygous.

The patient’s biosynthetic globin chain analysis showed two globin peaks eluting at the normal positions of β- and α-globin. Because the patient is homozygous, we infer that the variant globin chain elutes from the carboxymethylcellulose column (pH 6.32) at the same position as the normal β-globin chain. From quantitation of the radioactivity incorporated, a progressive deficit of β-globin chains was observed with increasing incubation time (Table 2). This result may be due to rapid degradation of abnormal β-chains.

Identification of the abnormal Hb. Sequencing of the β-globin gene showed a G to T base substitution within codon 12 (ie, GGC to GTG) that predicts that the glycine residue in this position is replaced by valine. This amino acid replacement has been previously reported in Hb Bushwick, an unstable variant. The sequence analysis indicated that the patient is homozygous for the mutation. The G to T base change causes the loss of an HaeIII restriction enzyme site. Using this enzyme, we confirmed that both parents, two siblings, the grandmother, and one aunt of the patient are indeed heterozygous for Hb Bushwick.

DISCUSSION

To the best of our knowledge, this is the first report of the homozygous state for an unstable Hb variant. One might classical Heinz bodies as well as very fine and numerous inclusion bodies in many of the red blood cells (RBCs). Other members of the family (Table 1 and Fig 3), apart from the patient’s sister (III-4), had normal or nearly normal Hb levels, normal RBC indices, and minimal morphologic abnormalities (Fig 2B). However, six of the eight members of the family were able to test (all except II-2 and III-3) had significant reticulocytosis. In all of these, Heinz bodies could be readily induced by incubating a peripheral blood sample at 50°C for 1 hour. Both glucose-6-phosphate dehydrogenase (G6PD) and 2,3-DPG levels were increased to an extent consistent with reticulocytosis.
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1979

Our patient’s condition had gone undetected until 13 years of age, although she was anemic, jaundiced, and below the 10th percentile in weight and height. The study of this family gave us an opportunity for the first time to compare the clinical expression of CHBA in a homozygote and in heterozygotes. In keeping with previous reports in the literature (Table 3),

The mildness of the heterozygous state is almost certainly related to the fact that the homozygous state is compatible with life and demonstrates the dosage effect in its expression.

We do not know whether the same would be true for Hbs that are more unstable. The closest match we could find in the literature is that of two reported cases of compound heterozygosity for Hb Duarte/β-thalassemia and Hb Köln/β-thalassemia. The 20-year-old man with Hb Duarte and β-thalassemia was investigated for darkening urine and jaundice, but was said to be ‘essentially asymptomatic,’ even though he had a markedly enlarged spleen. His Hb level was 15 g/dL and his reticulocyte count was 10.4%. The normal Hb may be attributed to the increased oxygen affinity of Hb Duarte ($P_50$ 16.03). The 35-year-old patient who was compound heterozygous for Hb Köln and β-thalassemia was reported to have tolerated her hemolytic anemia surprisingly well, requiring transfusions only on ‘occasions of various infections.’ Her clinical picture stabilized at 12 years of age, after which she required blood transfusion only once. Although our patient has also required blood transfusion only once, she does have chronic hemolytic anemia, which is susceptible to acute exacerbations; in at least one instance the anemia appeared to have been worsened by infection.

Laboratory diagnosis. The diagnosis of mildly unstable Hb variants is not easy, as highlighted by this case. The Hb electrophoretic pattern and the Hb A2 level were normal when a fresh sample was analyzed. A smear between Hb A and Hb A2 was only observed when a 24-hour-old sample was analyzed. The artefactual overestimation of Hb A2 is most probably due to heme depletion, thus confirming the instability of Hb Bushwick. We also note that Hb Bushwick had been classified as a slow-moving Hb, whereas its electrophoretic mobility is in fact normal, as expected for a Gly to Val replacement. The most likely explanation for this discrepancy is that what was reported to be the Hb variant was in fact a partially heme-depleted species. Furthermore, the heat stability test showed the presence of an abnormal Hb only after the incubation time had been extended from the normally recommended time of 1 hour to 3 hours. In light of this experience, we have adopted this prolonged incubation as part of our regular protocol when investigating unstable Hb and have not yet encountered false-positive results to date.

Pathophysiology. The nature of the patient’s anemia is certainly hemolytic, which is evident from the reticulocytosis...
and hyperbilirubinemia in the steady state. Hb Bushwick is relatively stable as a heme-containing tetramer, but once the heme group is oxidized it may be lost and then Hb Bushwick rapidly denatures. Thus, the stability of the molecule appears to be in a fine balance. This balance is tipped heavily when an infection or a drug causes oxidative damage, leading to severe hemolysis, as illustrated by our patient and in previous reported cases of individuals heterozygous for Hb Bushwick (Table 3). In view of our biosynthetic studies showing unstable \( \beta \)-globin chains, we suspect that the anemia is due in part to ineffective erythropoiesis.

**Molecular basis.** Glycine E18\( \beta \) \( ^{24} \) is an internal residue located in a surface crevice that lies between the E and F helices in the vicinity of, but not part of, the heme pocket. There are three known Hb variants, all unstable, in which this residue is affected. In order of increasing instability they are Hb Aalborg (Gly to Arg), \( ^{20} \) Hb Shepherds Bush (Gly to Asp), \( ^{21} \) and Hb Bushwick (Gly to Val). The introduction of an ionizable amino acid to the interior of the protein, such as aspartic acid or arginine, may cause some disruption of the heme-globin interaction. \( ^{22} \) However, these residues may still be accommodated in this position by the side chain facing out into the aqueous environment. \( ^{1} \) The milder phenotype of the Gly \( \rightarrow \) Arg substitution is attributed to the "greater externalisation of the ionisable group" in Hb Aalborg. \( ^{23} \) By contrast, the hydrophobic side chain of valine will
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Fig 3. Pedigree of family with Hb Bushwick. Solid or half-solid symbols indicate members homozygous or heterozygous for Hb Bushwick, respectively. Both first generation marriages (I-1 and I-2 also I-3 and I-4) are marriages between first cousins. Because II-2 and II-4 are heterozygous, we presume that I-3 is also heterozygous, like his brother (I-1). All heterozygotes have increased reticulocyte counts but this is much higher in the homozygous patient. NT, not tested.

Hb g/dL
Retics %

II

15.5
4.4
13.0
5.0
16.3
1.3
11.8
5.0

II

14.3
2.3

I

14.0
4.4
10.1
16.3
10.5
15.2
4.7

PATIENT

CONTROL

Hb A

Hb A2

Carbonic Anhydrase I

Carbonic Anhydrase II

Origin

Fig 4. Cellulose acetate electrophoresis (pH 8.5). The patient’s hemolysate on the left was deliberately overloaded to demonstrate the smeary band between the Hb A and HbA2, which explains the artefactually increased HbA2 levels.

Fig 5. Heat denaturation studies. Blood samples were incubated in Tris buffer (pH 7.4) at 50°C (Patient [□], mother [●], father [○], brother [▲], normal control [■]). After 5 hours, the unstable Hb was estimated to be 50% in the patient’s sample, 12% to 20% in heterozygous samples, and less than 3% in the normal control.
ACKNOWLEDGMENT

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