Hemoglobin North Shore: A Variant Hemoglobin Associated With the Phenotype of β-Thalassemia

By Clark M. Smith II, Bo Hedlund, John A. Cich, David P. Tukey, Mark Olson, Martin H. Steinberg, and Junius G. Adams III

Hemoglobin (Hb) North Shore (β34Val → Glu) is a mutant hemoglobin that is associated with the phenotype of mild heterogeneous β-thalassemia. Heterozygotes are characterized by low normal hemoglobin levels or mild anemia, microcytosis, increased HbA2, and 34%-38% Hb North Shore. The mechanism of the anemia and microcytosis associated with Hb North Shore was explored by studies of hemolyzate thermal instability, peripheral blood globin biosynthesis, and whole blood oxygen affinity. Hb North Shore was explored by studies of hemolysate mechanical variants that are poorly synthesized, leading to impaired synthesis rather than posttranslational degradation is responsible for the thalassemic phenotype associated with this variant hemoglobin. These observations parallel the findings in heterogeneous HbE. It is not presently known whether the thalassemia phenotype is conferred by the structural mutation itself or by another mutation cis to the β\textsuperscript{North Shore} gene.

MATERIALS AND METHODS

Standard hematologic techniques were used. Blood counts were performed with a Coulter model S plus electronic cell counter (Coulter Electronics Inc., Hialeah, Fla), and reticulocyte counts were determined by new methylene blue staining. Serum ferritin was determined by a Clinical Assays radioimmunoassay method (Gammapath \textsuperscript{21}Ferritin Radioimmunoassay Kit, Clinical Assays, Cambridge, Mass.). Hemoglobin electrophoresis was performed on cellulose acetate membranes at pH 8.6, citrate agar gels at pH 6.1, and by isoelectric focusing on thin-layer polyacrylamide gel plates. Hb North Shore was quantified by densitometric scanning of cellulose acetate gels and by DEAE Sephadex column chromatography. Fetal hemoglobin (HbF) was quantified by alkali denaturation and HbA\textsubscript{2} by microchromatography.

Oxygen Binding Studies

Whole blood oxygen binding data were obtained on the Hem-O-Scan Oxygen Dissociation Curve Analyzer (Aminco, Silver Spring, Md.) and also by the Instrumentation Laboratory Cobadetermining method (IL517 System, Instrumentation Laboratory, Lexington, Mass.). The pH of the Hem-O-Scan blood samples was determined by microelectrode after each run to standardize the oxygen tension to pH 7.4. Adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3DPG) was measured by the methods of Butler.

Hemoglobin Stability Studies

Whole blood was incubated with ascorbate and cyanide, and methemoglobin and sulfhemoglobin generation were measured. Hemoglobin stability was tested by heating at 50°C and precipitation in 17% isopropanol. The percent of hemoglobin precipitated by incubation at 60°C was determined from 2 to 30 min by the method of Huisman.

Reticulocyte Incubations

Blood was collected by venipuncture, anticoagulated with heparin, and immediately placed on ice. Packed red cells were obtained by centrifugation, diluted with two volumes of phosphate-buffered saline (PBS), and passed through a column of CF 11 cellulose (Ashless Fiber, Whatman, Clifton, N.J.) to remove white cells and platelets. After passage through the column, the red cells were spun down and washed twice with ice-cold PBS. In the pulse experiments,
Structural Identification

Purified abnormal β-chains were digested with trypsin as previously described. The tryptic peptides were separated on a 0.9 × 32 cm column of Beckman PA-35 resin, employing a gradient system described previously. Peptides were rechromatographed on a 0.9 × 50 cm column of AG 50W × 2 (Bio-Rad) following the procedure of Jones. A sample of each peptide was hydrolyzed in 5.7 M HCl under negative pressure for 22 and/or 72 hr at 110°C. The amino acid mixtures were analyzed on a Dionex amino acid analyzer kit with DC-4A resin and detected by o-phthalaldehyde fluorescence.

Amino acid sequence analyses were performed on a Beckman 890-C sequinator equipped with a cold trap attachment and Sequest T-6 auto converter. A program similar to that of Brauer et al., employing 0.3 M Quadrol, was used. The PTH-amino acids at each cycle of Edman degradation were identified by high performance liquid chromatography on a Vanlab 500 liquid chromatograph equipped with a Beckman-Altec dedicated PTH-amino reversed phase Ultrasphere-ODS column. Separation was achieved by the use of a linear gradient with an initial solvent of 0.42 M sodium acetate acids at some steps were carried out by isocratic runs using various proportions of the two solvents.

RESULTS

M.J. (III-1), a healthy 12-yr-old boy of Finnish extraction, was found to be mildly anemic on a blood count performed for suspected appendicitis. Subsequent evaluation discovered the presence of a mutant hemoglobin. There was no history of episodic pallor, jaundice, or left-sided abdominal pain. Exercise tolerance, growth, and development were normal. Physical examination was unremarkable and did not reveal a palpable spleen. His mother (II-5) had been treated for iron deficiency anemia in childhood and with each pregnancy. The pedigree of the family is shown in Fig. 1. The mutant hemoglobin must have been present in the maternal grandfather, who had affected children with two different wives.

Some hematologic features of the affected family members are presented in Table 1. All affected members had a low normal hemoglobin level or very mild anemia and, with the exception of II-2, all demonstrated microcytosis for age and decreased mean cell hemoglobin. Reticulocyte counts were minimally elevated in some individuals, but when corrected for hemoglobin level were not abnormal, and serum ferri-

### Table 1. Clinical Hematology of Family Members With Hb North Shore

<table>
<thead>
<tr>
<th>Member</th>
<th>Age (yr)</th>
<th>Hb (g/dl)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
<th>Reticulocytes (%)</th>
<th>Ferritin (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II-1</td>
<td>25</td>
<td>11.8</td>
<td>78</td>
<td>24.2</td>
<td>31.1</td>
<td>1.9</td>
<td>—</td>
</tr>
<tr>
<td>II-2</td>
<td>23</td>
<td>12.3</td>
<td>80</td>
<td>26.9</td>
<td>32.4</td>
<td>2.1</td>
<td>—</td>
</tr>
<tr>
<td>II-5</td>
<td>36</td>
<td>12.1</td>
<td>74</td>
<td>23.9</td>
<td>32.1</td>
<td>1.3</td>
<td>31</td>
</tr>
<tr>
<td>III-1</td>
<td>12</td>
<td>11.2</td>
<td>69</td>
<td>22.3</td>
<td>32.6</td>
<td>1.2</td>
<td>29</td>
</tr>
<tr>
<td>III-2</td>
<td>10</td>
<td>12.1</td>
<td>72</td>
<td>22.9</td>
<td>32.5</td>
<td>0.9</td>
<td>25</td>
</tr>
</tbody>
</table>

Some hematologic features of the affected family members are presented in Table 1. All affected members had a low normal hemoglobin level or very mild anemia and, with the exception of II-2, all demonstrated microcytosis for age and decreased mean cell hemoglobin. Reticulocyte counts were minimally elevated in some individuals, but when corrected for hemoglobin level were not abnormal, and serum ferri-
tin levels were normal when assessed. As noted in Fig. 1, Hb North Shore comprised 34%–38% of the total hemoglobin. All affected members had increased HbA₂ (normal 2.4% ± 0.8%), but the HbF was normal (<1%). Peripheral blood morphology showed subtle hypochromasia, microcytosis, and anisopoikilocytosis. White blood count and differential and platelet counts were normal. None of the affected members had abnormalities upon physical examination. None of the unaffected family members demonstrated clinical or laboratory abnormalities.

The mutant hemoglobin migrated as a band anodic to HbA upon alkaline cellulose acetate electrophoresis. It comigrated with HbA on citrate agar electrophoresis at pH 6.1. Isoelectric focusing demonstrated a hemoglobin band with an isoelectric point of 6.88 (HbA 6.95).

**Structural Studies**

Globin chain separation showed an abnormal β-globin peak eluting at a lower ionic strength than β⁺-globin (Fig. 2). The amino acid compositions of the peptides of the abnormal β-chain were normal, save for that of βT-14. The amino acid composition of βT-14 revealed a substitution of valine to glutamic acid or glutamine. Automatic sequence analysis demonstrated the sequence val-glu-ala-gly-val and, thus, a substitution of valine to glutamic acid at β134. This hemoglobin had previously been described as Hb North Shore and Hb North Shore/Caracas.¹⁵,¹⁶

**Hemoglobin Instability**

Incubation of whole blood from affected family members with ascorbate and cyanide for 1 hr resulted in a sulfhemoglobin precipitate without elevated methemoglobin. Hb North Shore hemolysates formed flocculent precipitates in a 1-hr incubation at 50°C and within 15 min during incubation in 17% isopropanol, whereas adult control hemolysates remained in solution. The percentage of hemoglobin progressively precipitated over a 30-min period by incubation at 60°C is shown in Fig. 3. The Hb North Shore hemolysates demonstrated greater thermal denaturation than the adult control hemolysate. The Hb North Shore instability was mild in comparison to other unstable hemoglobins evaluated in this laboratory (Hb Hammer-smith, Hb Kohn).

**Reticulocyte Biosynthesis**

In order to ascertain whether the mild instability of Hb North Shore was responsible for its low output, the proband’s reticulocytes were incubated with ³H-leucine for various times. Figure 2 shows chain separations or radioactive globin from 15-min and 120-min incubations. The radioactivity associated with β⁺ was half of the α-chain radioactivity that would be expected on the basis of gene dosage. The radioactivity associated with β North Shore is only about 25% that of the α-chain, which is about half that expected on the basis of gene dosage. The two radioactivity profiles are almost superimposable, suggesting that there is no degradation of β North Shore between 15 min and 120 min of incubation. The time course pulse incubations and pulse chase studies are shown in Fig. 4. The total β/α, β⁺/α, and β North Shore/α ratios did not change with time, indicating that β North Shore was not turned over during these experiments. Pulse–chase studies also demonstrate no change in the total β/α, β⁺/α, and β North Shore/α ratios. These results strongly suggest that decreased synthesis rather than posttranslational degradation of Hb North Shore was the significant factor in the reduced proportion of the variant hemoglobin in peripheral blood.
HEMOGLOBIN NORTH SHORE

Table 2. Hb North Shore Blood Oxygen Affinity

<table>
<thead>
<tr>
<th></th>
<th>$P_{50}$ (7.4)</th>
<th>2,3DPG</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(torr)</td>
<td>(M/gHb)</td>
<td>(M/gHb)</td>
</tr>
<tr>
<td>II-5</td>
<td>25.5</td>
<td>25.0-29.0</td>
<td>14.0-18.0</td>
</tr>
<tr>
<td>III-1</td>
<td>27.0</td>
<td>11-5 25.5</td>
<td>14.8 4.5</td>
</tr>
<tr>
<td>III-2</td>
<td>26.0</td>
<td>11-5 25.5</td>
<td>16.4 4.9</td>
</tr>
<tr>
<td>Normal</td>
<td>25.0-29.0</td>
<td>14.0-18.0</td>
<td>4.0-6.0</td>
</tr>
</tbody>
</table>

*$P_{50}$ determinations on two separate occasions.

Fig. 4. Globin synthesis ratios from intact reticulocytes in the pulse (upper panel) and the pulse-chase (lower panel) experiments. The details of these experiments are given in the text. The zero time point in the pulse-chase is a 15-min pulse.

Whole Blood Oxygen Affinity

The whole blood oxygen affinity, 2,3DPG, and ATP levels of Hb North Shore carriers were similar to normal adult controls, as shown in Table 2.

DISCUSSION

Hb North Shore is a $\beta$-chain variant that appears to be associated with the hemotologic phenotype of heterozygous $\beta$-thalassemia. Individuals heterozygous for Hb North Shore, like those with $\beta$-thalassemia trait, may have mild anemia, microcytosis, and an increased HbA$_2$ level. There was an imbalance in globin chain synthesis, with a deficit of $\beta$-chains relative to $\alpha$-chains. These results are unusual, since very few abnormalities of the primary structure of hemoglobin caused by single amino acid substitutions mimic the classical thalassemia syndromes. Leopre hemoglobin are synthesized in suboptimal amounts and produce a $\beta$-thalassemia phenotype. The $\alpha$-globin chain termination codon mutants are characterized by an elongated globin chain, unstable mRNA, and an $\alpha$-thalassemia-2 phenotype. Hemoglobin Indianapolis is synthesized at a near-normal rate, but its profound instability, with a half-life of less than 10 min, leads to a net synthesis of virtually zero and produces the findings of severe $\beta$-thalassemia. Hemoglobins E and Vicksburg are synthesized in reduced amounts associated with a decrease in $\beta$-chain mRNA.

Hb North Shore was slightly heat unstable and exhibited normal whole blood oxygen affinity as was previously reported. The substitution of a charged glutamic acid for a neutral valine at the internal $\beta$134 position might be expected to disrupt tertiary structure and lead to instability. The proximity of the substituted residue to the heme pocket did not appreciably alter whole blood oxygen affinity.

The synthesis of Hb North Shore $\beta$-chain was impaired in peripheral blood reticulocytes. It is unclear why our $\alpha/\beta$ ratios of about 1.35 differ from the more severely imbalanced ratios described previously. Our ratios were consistent at all time points in the pulse and pulse-chase experiments. Even at the shortest time point (15 min), the minimum number of counts in each of the peak tubes was 20,000. Although Hb North Shore is more heat unstable than HbA, this instability was not detectable in the course of globin biosynthesis studies and is quite mild when compared to other unstable variants. In one family member studied, there was no evidence of deletion $\alpha$-thalassemia by globin gene mapping (data not shown).

The anemia and microcytosis of Hb North Shore are most likely due to impaired synthesis of the mutant globin chain. The relative synthesis rates of mutant globins has been found to be reduced, albeit minimally, in a number of biosynthetic studies of unstable hemoglobins. Most of these studies did not evaluate very short incubation times or include pulse-chase experiments to separate globin instability from impaired synthesis. Unlike Hb North Shore, previous studies of unstable hemoglobin that have taken into account the possibility of globin instability have shown that the apparent alteration in synthesis was due to postsynthetic loss of the unstable globin.
While the instability of Hb North Shore might contribute to the microcytosis and mild anemia of the condition by slow precipitation of hemoglobin during the life of the red cell, it is more likely that the unbalanced globin chain synthesis is responsible for the hematologic findings and the phenotype of mild heterozygous thalassemia. Heterozygotes with Hb North Shore resemble individuals heterozygous for HbE. An individual who was a mixed heterozygote for HbS and Hb North Shore had the hematologic picture of HbS-β-thalassemia.16 Studies of mRNA levels and metabolism and the nucleotide sequence of the β North Shore gene may help clarify the reasons why this gene leads to mild β-thalassemia. It is not presently known whether the thalassemia phenotype is conferred by the structural mutation in the β-globin gene or by another mutation cis to the Hb North Shore gene.

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