Hemoglobin Duarte: \((\alpha_2^B \beta_2^A)\): A New Unstable Hemoglobin With Increased Oxygen Affinity

By E. Beutler, A. Lang, and H. Lehmann

A patient with a normal blood hemoglobin level, but with evidence of brisk hemolysis, was found to have an unstable hemoglobin with a left-shifted oxygen dissociation curve. Two sisters of the patient had a history of hemolytic anemia. Both died of thromboembolic complications following splenectomy. Our patient was found to be heterozygous for a new \(\beta\)-chain mutation and \(\beta\)-thalassemia. All of the hemoglobin in his red cells was of the abnormal type, designated hemoglobin Duarte, and was shown to contain proline at the \(\beta 62\) (E6) position instead of alanine.

A CONSIDERABLE NUMBER of unstable hemoglobins, which are associated with hemolytic anemia, have now been described. Because mutations that result in the formation of an unstable hemoglobin generally effect the binding of heme by globin, the contacts between the hemoglobin subunits, or disruption of the secondary structure of hemoglobin, they may result in an alteration of oxygen affinity. The combination of increased oxygen affinity and hemoglobin instability results in a hemolytic state in which the hemoglobin concentration of the blood is much higher than would be expected on the basis of the reduced life span of the erythrocytes. This combination—hemolytic anemia with a normal or near normal hemoglobin concentration of the blood—should suggest to the clinician the possibility that unstable hemoglobin with a left-shifted oxygen dissociation curve is present.

We have recently had the opportunity to examine a patient who presented this clinical picture. It is of interest that two sisters of the patient had been splenectomized at ages 19 and 20, and both had died within a year of surgery of thromboembolic complications.

CASE REPORT

C. B. is a 30-yr-old attorney who first noted darkening of the urine and jaundice at age 20. Similar episodes recurred from time to time, and he was referred to the City of Hope Medical Center in Duarte, Calif. because of a chronic hemolytic state that was, however, otherwise essentially asymptomatic.

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Red cell 2,3 DPG was 25 μmoles/g Hb. GSH was 4.99 μmoles/g Hb.

A pedigree is shown in Fig. 1. C.B. was one of four children. Two older sisters had been found to have enlarged spleens. One of the sisters was found to have a red count of 7,000,000/cu mm and a hematocrit of 52%. There had been two acute episodes in which the red cell count dropped precipitously with a concurrent reticulocytosis and jaundice. Osmotic fragility showed increased resistance to hemolysis. Because of the presence of hemolysis, splenectomy was carried out in 1947 when she was 19 yr old, and the patient died within a year following splenectomy, of embolic complications. The other sister was diagnosed, a few years later, as having hereditary spherocytosis. She was splenectomized and a few months later developed recurrent thrombophlebitis and multiple pulmonary emboli. In spite of anticoagulation, she died of pulmonary embolism some 9 mo following splenectomy. The parents of the propositus were of German origin. They were considered to be in good health, and two children were living and well.

An enlarged spleen, extending 8 cm below the left costal margin, constituted the only significant positive physical finding. The hemoglobin concentration of the patient's blood was 15.1 g/100 ml and the hematocrit 47%. The red count was elevated to 6.95 million, and a moderate degree of hypochromia and microcytosis was evident on the blood smear. The reticulocyte count was 10.4%. A 51Cr survival study gave a T1/2 of 16 days. The hematologic findings obtained on the patient and some of his relatives are summarized in Table I.

**MATERIALS AND METHODS**

Isopropanol stability tests were carried out by the method of Carrell and Kay; heat stability tests were carried out by the method of Dacie; oxygen dissociation curves were carried out by the mixing technique of Edwards and Martin, using five mix ratios in most cases, but only three ratios in the case of B.B. P50 values were corrected to pH 7.4, using the Bohr correction curves published by Röth. Hemoglobin was stripped of 2,3-DPG by dialysis for 28 hr against 0.05 M bis-Tris pH 7.35 containing 0.1 M NaCl, as described by Benesch and Benesch. Starch gel electrophoresis was carried out in Tris-EDTA-borate buffer, pH 8.8, in 10% electrostarch using the apparatus described by Smithies. Starch gel electrophoresis was also carried out in the discontinuous system described by Poulik, and paper electrophoresis was carried out as described by Craddock-Watson et al. Hemoglobin A2 determinations were carried out using a DEAE column. Red cell 2,3-DPG and GSH levels were assayed by previously described methods. Hemoglobin F levels were determined by alkali denaturation as described by Betke et al. Tests for inclusion bodies were made by incubating red cells with brilliant cresyl blue. Globin samples were prepared, both from the crude hemolysate and from the precipitate which formed with isopropanol, by the method of Anson and Mirsky and were separated into their constituent polypeptide chains by chromatography on carboxymethylcellulose (Whatman CMC-23).

**SPECIAL INVESTIGATIONS**

**Red Cell Metabolites**

Red cell 2,3 DPG was 25 μmoles/g Hb. GSH was 4.99 μmoles/g Hb.
Table 1. Hematological Findings

<table>
<thead>
<tr>
<th>Initials</th>
<th>Relationship</th>
<th>Pedigree Designation</th>
<th>Hb (g/100 ml)</th>
<th>Hct (%)</th>
<th>RCB (per cu mm)</th>
<th>MCV (μl/l)</th>
<th>MCH (μg/l)</th>
<th>MCHC (%)</th>
<th>Reticulocytes (%)</th>
<th>HbF (%)</th>
<th>HbA₂ (%)</th>
<th>Isopropanol Stability</th>
<th>Whole Blood</th>
<th>Inclusion Bodies</th>
<th>2,3-DPG (μmoles/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. B.</td>
<td>Propositus</td>
<td>II-4</td>
<td>15.1</td>
<td>47.4</td>
<td>6.95 x 10⁶</td>
<td>68</td>
<td>21.9</td>
<td>31.9</td>
<td>10.4</td>
<td>2.59</td>
<td>5.8</td>
<td>Pos</td>
<td>Pos</td>
<td>16.03</td>
<td>2.9</td>
</tr>
<tr>
<td>B. B.</td>
<td>Father</td>
<td>I-2</td>
<td>16.8</td>
<td>49.0</td>
<td>5.46 x 10⁶</td>
<td>90</td>
<td>30.8</td>
<td>34.3</td>
<td>0.6</td>
<td>0.48</td>
<td>3.4</td>
<td>Pos</td>
<td>Pos</td>
<td>0.0</td>
<td>Neg</td>
</tr>
<tr>
<td>I. B.</td>
<td>Mother</td>
<td>I-1</td>
<td>11.0</td>
<td>36.0</td>
<td>3.40 x 10⁶</td>
<td>67</td>
<td>20.3</td>
<td>30.5</td>
<td>1.2</td>
<td>0.50</td>
<td>4.9</td>
<td>Neg</td>
<td>Neg</td>
<td>27.04</td>
<td>2.9</td>
</tr>
<tr>
<td>V. T.</td>
<td>Sister*</td>
<td>II-3</td>
<td>14.8</td>
<td>42.6</td>
<td>12.6 x 10⁶</td>
<td>34.7</td>
<td>1.6</td>
<td>0.40</td>
<td>2.7</td>
<td></td>
<td></td>
<td>Neg</td>
<td>Neg</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>D. B.</td>
<td>Wife</td>
<td>II-5</td>
<td>13.3</td>
<td>40.2</td>
<td>4.61 x 10⁶</td>
<td>87</td>
<td>28.7</td>
<td>32.9</td>
<td>0.7</td>
<td></td>
<td></td>
<td>Neg</td>
<td>Neg</td>
<td>26.24</td>
<td>2.8</td>
</tr>
<tr>
<td>C. B., Jr.</td>
<td>Son</td>
<td>III-1</td>
<td>10.9</td>
<td>34.1</td>
<td>5.97 x 10⁶</td>
<td>57</td>
<td>18.2</td>
<td>32.1</td>
<td>1.2</td>
<td>1.04</td>
<td>5.7</td>
<td>Neg</td>
<td>Neg</td>
<td>2.9</td>
<td>Neg</td>
</tr>
<tr>
<td>D. B.</td>
<td>Son</td>
<td>III-2</td>
<td>9.6</td>
<td>31.2</td>
<td>5.43 x 10⁶</td>
<td>57</td>
<td>17.7</td>
<td>31.2</td>
<td>0.9</td>
<td>0.86</td>
<td>5.4</td>
<td>Neg</td>
<td>Neg</td>
<td>2.8</td>
<td>Neg</td>
</tr>
</tbody>
</table>

*Studies carried out by courtesy of Dr. William Larsen, University of Kansas.
Studies of Hemoglobin

Stability studies. The isopropanol stability test was consistently positive in 5 min when carried out on the patient's blood. The heat-stability test was also positive. Measurement of the supernatant indicated that only 7.8% of the hemoglobin was precipitated in 3 hr (control = 0.3%). On incubation of the blood of the propositus with brilliant cresyl blue, many of the red cells contained hemoglobin H-like inclusion bodies. As shown in Table 1, an unstable hemoglobin was also detected by the isopropanol stability test and the heat stability test in the patient's father, but not in his mother or two children.

Fetal hemoglobin, hemoglobin A2, free globin, and electrophoretic studies. The fetal hemoglobin concentration of the blood of the propositus was modestly elevated to 2.59%. All other family members studied had normal hemoglobin F values (Table 1). The hemoglobin A2 concentration of the patient's blood was elevated to 5.8%. As shown in Table 1, similar elevations were also found in the blood of both of his children and his mother, but his father and his wife had normal hemoglobin A2 concentrations. Except for the increase in hemoglobin A2 levels, no electrophoretic abnormality was noted in any of the electrophoretic systems. Examination of hemolysate prepared without organic solvents at 280 and 540 nm showed the presence of only 0.7% free globin.

Oxygen affinity studies. The whole blood of the patient displayed a marked left shift in the oxygen dissociation curve. The $P_{50}$ was only 16.0 mm Hg (normal = 26.7 mm Hg). This shift was all the more remarkable in view of the red cell 2,3-DPG level of 25.0 μmoles/g Hb, almost twice normal. Indeed, the hemoglobin, stripped of 2,3-DPG (2,3 DPG concentration = 0.07 μmoles/g Hb), had a $P_{50}$ of only 4.7 mm Hg by the mixing technique, approximately one-half of the normal value. The Hill constant was normal, at 2.9 for whole blood and 2.7 for the stripped hemoglobin solution. Fresh blood from the patient's father was not available for oxygen affinity studies, but stripped hemoglobin from the father also revealed a moderately increased oxygen affinity. In contrast, the oxygen affinity of the whole blood of the patient's two sons was normal (Table 1).

Structural studies. The elution profile of the globin sample prepared from unpurified hemolysate confirmed a significant increase in the proportion of β-chains, and hence Hb A2, in the hemolysate but did not indicate which polypeptide chain was abnormal. The elution profile of the globin prepared from the isopropanol precipitate, however, indicated a marked difference in the amounts of α- and β-chains in the precipitate. Quantitation of the isolated chains by weight showed that there were approximately twice as many β-chains present as α-chains. Since it has been reported¹ that precipitation with isopropanol produces a preponderance of β-chains in precipitates of several unstable hemoglobins with abnormal β-chains, it seemed probable that the mutation responsible for the instability of this hemoglobin had also occurred in the β-chain.

The fractions corresponding to the β-chain prepared from the hemolysate were pooled. The solution was made 1 M with respect to Tris and the pH adjusted to 9.3 with concentrated HCl before reacting with ethylene imine. The aminoethylated β-chain was then isolated by lyophilization after extensive
dialysis of the solution against 0.5\% formic acid at 4°C to remove all traces of urea and salts. Fingerprints of the tryptic digests of the aminoethylated β-chain were prepared and stained with ninhydrin and other reagents specific for the amino acids arginine, histidine, methionine, tryptophan, and tyrosine. The pattern of the peptides on the fingerprint (Fig. 2) did not suggest an obvious change in either the composition or position of any of the peptides, although the area of the fingerprint corresponding to β TpVI appeared more distorted than normal. This area was, therefore, rerun at pH 3.5 for 1 hr at 2.5 kV. The purified peptide was eluted with 6 N HCl, hydrolyzed for 24 hrs at 108°C, and analyzed in a Locarte amino acid analyzer. The amino acid composition of this peptide did not, however, correspond to β^A Tp VI.* In fact, a peptide with the composition of β^A TpVI (residues β60–61 Val-Lys) could not be located on the fingerprint of the aminoethylated β-chain from either the hemolysate or the precipitate. This indicated that no normal β-chains were present. Furthermore,

*As mentioned above, in the fingerprint of the aminoethylated β-chain from Hb Duarte, another peptide was found in the position normally occupied by β^A TpVl. The amino acid composition of this peptide corresponded to that expected for the sequence β140–144 of the normal β-chain and is shown in Table 2. The yield of the peptide (18.1 nmoles) and the mean yield of five other peptides from the same fingerprint (57.3 nmoles) requires that 30\% of the available Asn-Ala bonds (β138–139) are hydrolyzed during trypsin hydrolysis.

The peptide cannot be derived from a contaminating protein, since it has been found in trypsin digests of isolated β-chains from purified hemoglobins. Furthermore, in a recent sample of Hb K Woolwich (β132 Lys → Glu), a negatively charged tryptic peptide was isolated from the fingerprint of the purified β^K-chain which had an amino acid composition corresponding to the sequence β^K121–139. Since these peptides were obtained with two commercial preparations of trypsin [recrystallized trypsin and (l-tosylamido)-phenylchloromethylketone (TCPK) treated trypsin from Worthington Biochemical Corporation, N.J.], it would appear that trypsin hydrolyses nonspecifically the bond between β138 asparagine and β139 alanine.
the absence of βTpVI also serves to limit the number of likely substitutions. Since the hemoglobin showed the same electrophoretic mobility as Hb A, it was unlikely that the β61 lysine involved in the tryptic hydrolysis was itself missing. A mutation of the β60 Val to another neutral amino acid was an unlikely explanation for the findings, since this would not appreciably have altered the chromatographic mobility of the βTpVI peptide, and one would have found it in the expected position. Deletion of the β60 Val would have produced a loss of the βTpVI peptide, but this seemed highly unlikely, because the deletion of this important residue would cause much more disturbance in the hemoglobin than was observed. The most likely possibility seemed to be that the mutation involved the residue following the lysine, namely that β62 Ala had mutated to proline. This mutation is permitted by a single change in the genetic code, and it would explain the absence of the βTpVI peptide, since proline inhibits tryptic hydrolysis. It was deduced that the resulting peptide 60-65 (β62 Ala→Pro) would migrate on electrophoresis chromatography with β^A TP VII. This area was therefore cut from a preparative fingerprint and purified by two-dimensional electrophoresis chromatography (electrophoresis at pH 6.5 for 30 min at 3.0 kV and ascending chromatography for 20 hr in butanol:acetic acid:water, 60:15:25). Two major peptides were located by ninhydrin, and both were eluted, hydrolyzed, and analyzed as before. The faster migrating peptide gave only lysine on analysis and must correspond to β66 Lysine (βTpVIII). The other peptide had the amino acid composition shown in Table 3. On comparison with the composition expected for β^A TP VII, this peptide had no alanine and additional residues of valine, proline, and lysine. The most likely explanation was, as mentioned above, that β62 alanine had been replaced by a residue of proline, since the resulting Lys-Pro peptide bond (β61–62) would not be hydrolyzed by trypsin.16 This would result in the appearance of a tryptic peptide with the composition shown in Table 3 and the sequence shown in Fig. 3. To prove this conclusively, the sequence of the first three residues of the peptide

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Hb Duarte (β60–65)</th>
<th>Molar Ratios</th>
<th>Hb A (β60–65)</th>
<th>Expected Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro</td>
<td>10.96</td>
<td>0.90</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>14.29</td>
<td>1.11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>13.81</td>
<td>1.06</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>12.65</td>
<td>0.98</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>24.98</td>
<td>1.93</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Amino Acid Composition of the Peptide That Migrated as β^A TP VI in the Peptide Map of the Tryptic Digest of the Aminoethylated β-chain of Hb Duarte
was investigated by dansyl-Edman degradation. The peptide was purified as before from several preparative fingerprints and eluted with 0.5 N NH₄OH. The isolated peptide was divided into three aliquots. The first was dansylated directly, while the second and third samples were subjected to one and two steps of Edman degradation, respectively, before dansylation. The dansyl derivatives of the first three residues of the peptide were identified by thin layer chromatography as DNS valine, bisDNS lysine, and DNS proline, indicating that the sequence of the peptide is that shown in Fig. 2. The unstable hemoglobin has, therefore, an abnormal chain with the mutation (E₆) Ala→Pro. This variant has not been described before and has been given the name hemoglobin Duarte.

DISCUSSION

Unstable hemoglobins are inherited as autosomal dominant disorders. The existence of clinically significant hemolytic disorders in the patient and his sisters, but in none of the parents or the children of the propositus, therefore seemed to be somewhat surprising. When the patient was originally studied, he was noted to have mild hypochromia, microcytosis, and elevated hemoglobin A₂ levels. We initially considered all of these to be secondary manifestations of the inheritance of an unstable hemoglobin. When his children were examined, however, it became apparent that they had inherited the microcytosis and hypochromia, as well as elevated hemoglobin A₂ levels, but not the abnormal hemoglobin. Since the children’s mother was normal, it became apparent that the propositus actually carried two genes: the gene for thalassemia and that for an unstable hemoglobin. Examination of the blood of his parents revealed that his father had the unstable hemoglobin, while his mother had the β-thalassemia trait. His children, apparently, had only inherited the β-thalassemia gene. Structural studies of the hemoglobin confirmed this interpretation. While patients heterozygous for an unstable hemoglobin gene and a normal hemoglobin gene invariably have, in addition to the unstable hemoglobin, a large amount, usually more than one-half, of normal hemoglobin, C.B. was found to have no normal β-chains. Thus, the thalassemia minor gene was apparently a “severe,” β⁰ type.

The residue β62 (E₆) Ala affected in Hb Duarte is an external residue in the normal β-chain and is, therefore, not a heme contact, nor is it a subunit contact. However, it participates in maintaining the E helix, and several residues in this helix are heme contacts, e.g., E7 His, E10 Lys, E11 Val, and E14 Ala. Since proline can only be tolerated as one of the first three residues of a regular helix, the substitution of E6 alanine by proline must be expected to cause disruption of this helix. While it is impossible to predict the extent of this disrup-
tion, it seems reasonable to assume that it could affect some or all of the heme contacts mentioned above. However, the weakly positive isopropanol stability test in the propositus and the heterozygote for Hb Duarte and Hb A does suggest that the instability caused by the mutation is not particularly deleterious. Nevertheless, the increased oxygen affinity of the hemoglobin is indicative of changes occurring in the heme pocket, which allow easier access of oxygen to the iron atom.

It is clear that the β62 (E6) alanine → proline substitution must have relatively mild effects. The heterozygous father of the propositus appeared normal hematologically. If a β-thalassemia gene had not been inherited together with the gene for Hb Duarte, the abnormal hemoglobin would probably never have been noticed. Even the complete replacement of β-chains by the mutant chain caused only a relatively benign disorder. Serious thromboembolic complications occurred in the patient's sisters only following splenectomy, which may have resulted in erythremia and thrombocytosis. It should be emphasized that most patients with an unstable hemoglobin with increased O2 affinity, who have undergone splenectomy, have not developed dire complications. Thrombocytosis and thromboembolic complications have been documented in some patients who have undergone splenectomy because of unstable hemoglobinopathies; some patients, on the other hand, appear to have benefited from the procedure. The tragic apparent consequence of splenectomy in these two girls indicates the need for caution in splenectomizing such patients and emphasizes the importance of accuracy of laboratory diagnosis of hemolytic disorders whenever possible. Unfortunately, unstable hemoglobins and hemoglobins with altered oxygen-binding properties had not yet been discovered when the sisters of the patient were first studied.

ACKNOWLEDGMENT

We are grateful to Dr. Gurth Carpenter for referring this interesting family to us for study and to Mrs. Carol West for valuable technical assistance.

REFERENCES

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