Hemoglobin-Seattle \((\alpha^A\beta^A_{76}\text{Glu})\): An Unstable Hemoglobin Causing Chronic Hemolytic Anemia

By E. R. HUEHNS, F. HECHT, A. YOSHIDA, G. STAMATOYANNOPoulos, J. HARTMAN and A. C. MOTULSKY

Mild to moderate chronic hemolytic anemia in a mother and her two sons was found to be associated with the presence of about 40 per cent abnormal hemoglobin of rapid electrophoretic mobility: Hb-Seattle. Hb-Seattle was found to be unstable in vitro. It was shown to be due to a replacement of alanine by glutamic acid at position 76 of the \(\beta\) hemoglobin chain. The substitution occurs at the surface of the \(\beta\) hemoglobin chain. The hemoglobin instability was attributed to an abnormal interaction between the substituted glutamic acid and histidine at position \(\beta^{77}\).

RECENTLY, a number of patients have been described who suffer from hemolytic disease due to an unstable hemoglobin in the heterozygous state. The first of these was Hb-Zurich disease.\(^1,2\) Since then, several other such hemoglobins have been reported.\(^3,4\) In this paper, the occurrence of a mild chronic hemolytic anemia in association with the heterozygous state of an unstable abnormal hemoglobin, Hb-Seattle, is reported.

METHODS AND RESULTS

The Proposita

The patient, a 28-year-old white woman, was first noted to be anemic at age seven (1941) when she was hospitalized for burns. At about 17 years of age the anemia became more severe, and she was given two blood transfusions. At 20 years of age, her hemoglobin was 9.7 Gm. per 100 ml., and although the findings were not typical, the diagnosis of "thalassemia minor" was suggested. The patient was brought to our attention in 1962, because one of her children was also found to be anemic. Hematological data are shown in Table 1.

The patient had never had any abdominal or other pain. She had not been jaundiced or passed red or brown urine. Physical examination was normal. The liver and spleen were not palpable. X-rays of the skull and hands were normal.

The patient was reexamined in 1967. During the intervening five years, she had not

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STATEMENTS OF AFFILIATION

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Blood, Vol. 36, No. 2 (August), 1970
Table 1.—Hematologic Values of the Proposita and Her Two Sons

<table>
<thead>
<tr>
<th></th>
<th>Proposita</th>
<th>S.S. (6 yrs.)</th>
<th>R.S. (5 yrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (Gm./100 ml.)</td>
<td>10.4</td>
<td>9.8</td>
<td>9.5</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>31.5</td>
<td>27.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Red Cell Count (× 10⁶/cu.mm.)</td>
<td>3.62</td>
<td>3.29</td>
<td>3.32</td>
</tr>
<tr>
<td>MCV (µ₃)</td>
<td>87</td>
<td>82</td>
<td>75</td>
</tr>
<tr>
<td>MCH (in γ per red cell)</td>
<td>29</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>MCHC (Gm./100 ml.)</td>
<td>33</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>3.0</td>
<td>2.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Red cell morphology</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>White cell count</td>
<td>11,400</td>
<td>7300</td>
<td>3600</td>
</tr>
<tr>
<td>Platelet count</td>
<td>255,000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Plasma iron (µg./100 ml.)</td>
<td>117</td>
<td>108</td>
<td>100</td>
</tr>
<tr>
<td>TIBC (µg./100 ml.)</td>
<td>297</td>
<td>295</td>
<td>278</td>
</tr>
<tr>
<td>Hb Seattle %</td>
<td>43.0</td>
<td>39.0</td>
<td>42.0</td>
</tr>
</tbody>
</table>

suffered from any symptoms due to her chronic hemolytic anemia. However, she gave a history of increased blood loss during menstruation, and examination showed that she had developed iron deficiency anemia. After treatment with oral iron her hematological measurements returned to those found in 1962.

Family Study

The patient was adopted as an infant. Study of her two children (R.S., aged 5 years; S.S., aged 6 years) showed that both carried the abnormal hemoglobin and also suffered from the same type of mild hemolytic anemia (Table 1). The patient’s husband was normal on clinical and hematological examination.

Hematological Study

When first examined, the bone marrow in the proposita was normocellular with slightly increased erythroid-myeloid ratio. Iron stores appeared adequate. The time for 50 per cent disappearance of Cr⁵¹ labeled red cells was 16 days (normal 25–30 days). In a cross-transfusion experiment, when red cells containing Hb-Seattle were given to a normal individual, the cell survival was reduced with a value similar to that in the patient. In neither study was there any detectable accumulation of Cr⁵¹ in the spleen. There was a twofold increase in values of plasma iron turnover⁵ (proposita: 1.21 µg./100 ml. of whole blood/day; normal values: 0.7 µg./100 ml. whole blood/day) and erythrocyte iron turnover (proposita: 1.03 µg./100 ml. whole blood/day; normal values: 0.56 µg./100 ml. whole blood/day).

Direct Coomb’s test was negative. Osmotic fragility and incubated (24 hours) osmotic fragility tests were normal. The results of the auto-hemolysis test⁶ were all normal: at 37° C: 1.9 per cent hemolysis without substrate, 0.7 per cent hemolysis with glucose, 0.9 per cent hemolysis with inosine; at 4° C: 0.2 per cent hemolysis without substrate, 0.0 per cent hemolysis with ACD, 0.2 per cent hemolysis with EDTA.

Red cell ATP was 4.36 µmoles/Gm. Hb (4.04 ± 1.26); ADP was 0.52 µmoles/Gm. Hb (0.57 ± 0.32); GSH was 5.5 µmoles/Gm. Hb (7.5 ± 2.6); GSH stability test: 74 per cent (91 ± 13); G6PD was 6.7 U/Gm. Hb (4.87 ± 0.24); 6PGD was 3.2 U/Gm. Hb (2.97 ± 0.22). These investigations on the red cells were made as described in Reference 6 and the means of normal values plus two standard deviations are given in parentheses.

The serum bilirubin in the proposita was 0.2 mg. direct and 0.6 mg./100 ml. total, and the urine urobilinogen was 0.4 Ehrlich units/100 ml. A battery of clinical chemical tests (BUN, uric acid, thymol turbidity, alkaline phosphatase, SCOT, SCPT, serum protein electrophoresis and urine guaiac) were all within normal limits.
Demonstration of Hemoglobin Instability

Inclusion bodies. In unstained fresh red cells no inclusion bodies were seen either by bright field or phase contrast microscopy or after staining with 1 per cent brilliant cresyl blue. Metemoglobin in fresh red cells was less than 1 per cent of total hemoglobin. However, when the patient's red cells were incubated at 37°C in a sterile, isotonic phosphate buffer, pH 6.4, without glucose, dark staining inclusion bodies were seen after 12 hours of incubation. They increased in frequency until the end of incubation at 24 hours. Normal cells showed an occasional inclusion body after 24 hours of incubation, but the difference between normal cells and the patient's cells was marked (Fig. 1).

Methemoglobin formation. Under the same conditions of incubation, the patient's red cells accumulated methemoglobin more rapidly than normal cells; at 24 hours, 23 per cent of total hemoglobin had been converted to methemoglobin. Red cells containing only hemoglobin A or hemoglobins A + S or A + C or A + Tacoma always formed less than 8 per cent of methemoglobin when incubated under the same conditions for 24 hours. Red cells containing hemoglobins A + H formed 17 per cent of methemoglobin.

The in vitro instability of Hb-Seattle was demonstrated by the following findings:

Heat precipitation. Hemoglobin heat instability was assessed after heating the hemolysates for two minutes at several temperatures from 37°C to 100°C and plotting the per cent precipitations against temperature. In hemolysates from the patient 50 per cent precipitation had occurred at 72°C, while with normal hemolysates this did not occur until 80°C. Starch gel electrophoresis of hemolysates containing Hb-Seattle indicated that heating for two minutes at 75°C led to complete precipitation of Hb-Seattle, while neither Hb-A nor Hb-A₂ was noticeably affected (Fig. 2). In incubations of hemolysates at 60°C, hemoglobin denaturation in hemolysates containing Hb-Seattle was first observed after one hour; the curve expressing the proportion of undenatured hemoglobin against time...
Fig. 2.—Heat denaturation of Hb-Seattle. Note the disappearance of Hb-Seattle from the hemolysate while Hb-A and Hb-A₂ are not affected. Starch gel electrophoresis in a tris-EDTA-borate buffer pH 8.6.

was shifted to the left of the normal curve by one and a half hours in the hemolysate containing Hb-Seattle. These results indicate that Hb-Seattle is more easily denatured by heat than Hb-A.

Storage. Exposure of pH 4.7 for 16 hours led to complete precipitation of Hb-Seattle. Storage of hemolysates containing Hb-Seattle in the refrigerator showed a gradual decrease in the proportion of the abnormal hemoglobin, and only small amounts could be detected after two to three weeks. This relatively rapid denaturation and precipitation of Hb-Seattle could be prevented in solutions containing 0.01 M KCN. In solutions of isolated Hb-Seattle, a fine precipitate gradually appeared on storage. Starch gel electrophoresis of the supernatant hemoglobin solutions showed some free normal α-chain hemoglobin.

Characterization of Hb-Seattle

Electrophoretic Characterization. Paper, starch block, and moving boundary electrophoresis in barbiturate buffer pH 8.6, 0.5 M, as well as starch gel electrophoresis using a tris-citrate/borate discontinuous buffer system at pH 8.6, did not resolve the abnormal hemoglobin from Hb-A. Hemoglobin-Seattle was, however, easily resolved on starch gel, both in a tris-EDTA-borate buffer, pH 8.6 (Fig. 2) and in a phosphate buffer, pH 7.4, as well as on starch block electrophoresis using a 0.04 M sodium phosphate buffer, pH 7.0. In all these buffer systems Hb-Seattle migrated like Hb-J. The proportion of Hb-Seattle, measured by starch block electrophoresis was 43 per cent. The level of Hb-A₂ was 2.5 per cent and that of fetal hemoglobin was normal.

The properties of the oxy- and carboxy-hemoglobin or the derivative of methemoglobin on starch block and starch gel electrophoresis were identical. However, methemoglobin Seattle could not be resolved from methemoglobin A in any of the three buffer systems used on starch gel electrophoresis.

Chromatographic Separation. Hb-Seattle could not be resolved from Hb-A by chromatography on carboxy-methyl-Sephadex (Sephadex CM 50) while excellent separations were obtained with DEAE-Sephadex columns using a tris-HCl 0.01 M buffer and a pH gradient 8.2 to 7.3. The proportion of Hb-Seattle, by this technique, was 42 per cent in the proposita and 39 per cent and 44 per cent in sons S.S. and R.S., respectively.

Hybridization Studies. Hybridization studies were carried out as described in Reference...
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Fig. 3.—Comparison of the fingerprints prepared from the tryptic hydrolysates of Hb-Seattle and Hb-A; high voltage electrophoresis in pyridine: acetic acid:water (10:0.4:90) buffer, pH 6.4, followed by ascending chromatography in isoamyl alcohol-pyridine-water (35:35:27). Note the replacement of $\beta^8T$-9 and $\beta^4T$-8+9 by two new peptides, $\beta^{Seattle}T$-9 and $\beta^{Seattle}T$-8+9 with a greater anodic mobility.

9, using pH 4.7 for the exchange of subunits. Approximately half the hemoglobin present precipitated during the experiment. Starch gel electrophoresis of dissociated-recombined Hb-Seattle and Hb-Canine showed that, besides canine hemoglobin, the hybrid $\alpha_2\beta_2^{Can}$ could be recovered in good yield. Hb-Seattle and the other expected hybrid $\alpha_2\beta_2^{Seattle}$ were only present in trace amounts. This experiment indicated that Hb-Seattle contained normal $\alpha$-chains and, therefore, had the composition of $\alpha_2\beta_2^{Seattle}$.

Absorption Spectra. The absorption spectra were measured on a Beckman model DK2 recording spectrophotometer. The positions of the heme absorption maxima and minima of the carbonmonoxo-, cyanmet- and met-derivatives of Hb-Seattle were identical to those of the corresponding derivatives of Hb-A; the absorbance ratios between the maxima and minima of the carbonmonoxo- or the cyanmet-derivatives of Hb-A and Hb-Seattle were also identical. However, the absorption spectrum in the region of 550–650 m$\mu$ of the acid met-derivative (measured in a M/15 sodium phosphate buffer, pH 6.5) of Hb-Seattle differed significantly from that of the same derivative of Hb-A. The inflexion present in Hb-A at ca 575 m$\mu$ was almost completely absent in Hb-Seattle. The absorbance ratios, 500 m$\mu$/630 m$\mu$ were significantly different, being 2.51 for Hb-A and 2.82 for Hb-Seattle; the fractional resolution, $\Delta$, of the maximum at 630 m$\mu$

\[
\Delta \text{ OD max at } 630 = \frac{\text{OD max at } 630 - \text{OD min at } 600}{\text{OD max at } 630}
\]

was 0.18 for met-Hb-Seattle and 0.28 for met-Hb-A. The rate of reaction with cyanide of the methemoglobin derivative was less than 15 seconds for complete reaction, i.e., indistinguishable from that of met-Hb-A.

Molecular Size of Hb-Seattle. Sedimentation studies were carried out in a Spinco Model E ultracentrifuge equipped with an RTIC unit with Schlieren optics at 10° C. A type AN-D rotor was used. The sedimentation coefficient ($S_{20, W}$) of the cyanide derivative
Fig. 4.—Elution patterns of tryptic hydrolysates of normal and Hb-Seattle β-chains from a Spinco 15-A resin column (0.9 x 17 cm.). The peptides were eluted from a linear gradient produced by adding 300 ml. of 2 M pyridine-acetic acid buffer, pH 5.0, into a mixing chamber containing 30 ml. of 0.2 M pyridine-acetic acid buffer, pH 3.1. The flow rate was 35 ml./hr. at 50°C. The effluent stream was split; about one-fifth was used for continuous detection by reaction with ninhydrin after hydrolysis with 4 N NaOH, and the remainder was collected in a fraction collector. Peptides are numbered in accordance with Jones. The second half of the elution patterns is not shown.

of methemoglobin-Seattle in a 0.015 M sodium phosphate buffer (containing 0.01 M KCN) at pH 6.8 and a protein concentration of 13.9 mg. per milliliter was 4.07 S. This result is within the range of S values obtained for normal adult hemoglobin and therefore indicates that Hb-Seattle, like Hb-A, consists of four polypeptide chains. Further confirmation that Hb-Seattle has the same molecular size as Hb-A comes from the elution pattern obtained from Sephadex G-100. Using the experimental conditions already described, Hb-A and Hb-Seattle were eluted with the same buffer volume.

Structural Studies

Tryptic peptide maps were made as previously described and that of Hb-Seattle is compared with that of Hb-A in Fig. 3. It can be seen that peptide βT9 is replaced by a new, more negatively charged, peptide. Similarly, peptide βT8+9 is replaced by a neutral peptide.

For further studies, the β-chain of Hb-Seattle was isolated by column chromatography of the Seattle globin, as described by Clegg, Naughton and Weatherall. The isolated β-chain was aminoethylated and digested with trypsin, and the peptides were separated by automatic column chromatography. The elution profile of the tryptic peptides from S-aminoethylated β-chain of Hb-Seattle is shown in Fig. 4. When compared with the pattern for the normal β-chain, βT-9 and βT-8+9 of Hb-Seattle were eluted slightly earlier. The abnormal peptides from these peaks, after rechromatography and hydrolysis in 6 N HCl, gave the amino acid composition shown in Table 2. The results indicated that one of the two alanine residues (position 70 or 76) was replaced by glutamic acid or glutamine.

After four steps of Edman degradation, using a small scale modification of the method of Konigsberg and Hill, of the N-terminal end of βT-9 of Hb-Seattle, alanine diminished while glutamic acid (or glutamine) remained (Table 2). This fact indicated that position
Table 2.—Amino Acid Composition of Tryptic Peptides of \(\beta\)-chain of Hb-Seattle Before and After Edman Degradation

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal βT-9</th>
<th>βT-8+9† Before Edman</th>
<th>Hb-Seattle Before Edman</th>
<th>Hb-Seattle After 3rd Edman</th>
<th>Hb-Seattle After 4th Edman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Serine</td>
<td>1</td>
<td>1.1</td>
<td>1.2</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>2.0</td>
<td>2.0</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>2</td>
<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Valine</td>
<td>1</td>
<td>1.1</td>
<td>0.9</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Leucine</td>
<td>4</td>
<td>3.6</td>
<td>3.8</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1</td>
<td>0.8</td>
<td>1.0</td>
<td>0.7</td>
<td>†</td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>0.9</td>
<td>1.1</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td>Lysine</td>
<td>1</td>
<td>1.8</td>
<td>1.0</td>
<td>†</td>
<td>†</td>
</tr>
</tbody>
</table>

† Ratio of amino acids of purified \(\beta\)-8,9 and \(\beta\)T-9 peptides from column chromatography, taking aspartic acid as 3.0.
† Not determined.

70 in Hb-Seattle was alanine. Consequently, these results show that the alanine residue at position 76 was replaced by glutamic acid (or glutamine) in the \(\beta\)-chain of Hb-Seattle.

Since the abnormal peptides (\(\beta\)T-9 and \(\beta\)T-8+9 from Hb-Seattle) contain one more negative charge at pH 6.4 than those from normal hemoglobin, the new amino acid must be glutamic acid. In order to confirm this directly, the abnormal peptide \(\beta\)T-9 was digested by leucine amino peptidase, carboxy-peptidase A and carboxy-peptidase B, and the liberated amino acids were identified.16 This showed that the peptide contained glutamic acid, not glutamine, indicating that the structure of Hb-Seattle is \(a_2\beta_2\). This substitution is consistent with a one-step mutation. The only two code words for glutamic acid are GAA and GAG, while alanine is coded for by GCU, GCC, GCA and CCC. The postulated mutational step in Hb-Seattle, therefore, was a \(G_{\beta 76} \rightarrow \Delta_{\gamma}\) transversion.

**Discussion**

The hemolytic anemia seen with Hb-Seattle in all likelihood is caused by the presence of this unstable hemoglobin. Generally, however, with only a mild reduction in red cell life span, there should be erythropoietic compensation and a normal hemoglobin level. Recent studies17,18 have shown that release of erythropoietin and the consequent erythropoietic marrow response depend upon the patterns of the hemoglobin-oxygen equilibria. Several abnormal hemoglobins—Hb-Chesapeake,19 Hb-Yakima,20 Hb-Kempsey,21 and Hb-Rainier22—release a smaller proportion of oxygen per red cell to the tissues when compared with normal. The resulting tissue hypoxia stimulates erythropoietin secretion and this leads to erythrocytosis. The oxygen dissociation of several unstable hemoglobins resembles that of the “polycythemic” hemoglobins21 so that maximal erythropoietic marrow response occurs in these instances. Studies on the oxygen equilibrium and erythropoietic responses in patients with Hb-Seattle18 indicated decreased oxygen affinity with nearly normal heme-heme interactions, so that in contrast to the hemoglobins mentioned above, a greater proportion of oxygen per red cell is released to the tissues. Under these condi-
tions, a lessened erythropoietic response would be expected; in fact, the measured erythropoietin levels in the patients with Hb-Seattle was minimal for the levels of their hematocrit. Thus, the anemia in patients with hemoglobin Seattle is mainly caused by lessened erythropoietin secretion due to the increased release of oxygen by the abnormal pigment. The latter situation has also been observed in hemoglobin E disease.

Recent work on the three-dimensional structure of proteins has given insight into the type of amino acid substitutions which may give rise to instability of the hemoglobin molecule. Most previously reported unstable hemoglobins have either been substitutions on the interior of a subunit, at a heme contact or at one of the intersubunit contacts. Of the more than 50 substitutions known to occur at the surface of the molecule, only one (i.e., that in Hb-Sealey-Sinai-Hasharon) is associated with instability and mild hemolytic disease. Hb-Seattle is also unusual in that an amino acid substitution at the surface of the molecule causes molecular instability and an altered oxygen affinity.

Examination of the atomic model of hemoglobin shows that the new glutamic acid introduced at $\beta$-76 is close to the histidine $\beta$-77. The introduction of a negative charge at position $\beta$-76, on the surface of the molecule, may cause a change in the three-dimensional arrangement of the affected subunit, probably through an interaction with the histidine at $\beta$-77. This interaction possibly acts through an “effector” (such as, for example, 2-3 diphosphoglycerate) leading to low oxygen affinity without gross change in heme-heme interaction. It could also lead to increased dissociation into subunits followed by increased precipitation of the unstable $\beta$-Seattle chain. The electrophoretic properties of hemoglobin Seattle lend some support to this hypothesis. In phosphate buffer near neutral pH, histidines would normally be expected to be charged and interaction with the new glutamic acid at $\beta$-76 would lead to the loss of one positive charge per $\beta$-chain, and the observed increased electrophoretic mobility. At pH 8.6, well above the pK of histidine, this amino acid would normally carry no charge. However, when a glutamic acid is introduced close to histidine $\beta$-77, it increases the pK of the latter. Thus, at pH 8.6 the histidine remains positively charged, it neutralizes the new acidic group, and no change in net charge of the molecule nor electrophoretic separation should occur. In fact, this was the finding when barbiturate or Tris-citrate/borate buffer electrophoresis was performed at pH 8.6.

In interpreting the importance of an amino acid substitution at any one site, the variability of this site between species has also to be taken into account. $\beta$-76 is a variable residue; lys, ala, glx (presumably glutamine), his, gly, ser, asn, having been found occupying this structural site. Although both neutral and basic residues occur at this site, acidic residues have so far not been observed in normal hemoglobins. It is thus probably of significance that the only hemoglobin to be observed with an acidic residue at this site, Hb-Seattle, was also functionally abnormal.

*However, no immediate explanation is apparent for the results obtained on starch gel electrophoresis in tris-EDTA-borate buffer pH 8.6 as well as for the electrophoretic behavior of the methemoglobin derivative of Hb Seattle.
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ACKNOWLEDGMENTS

We are grateful to Dr. E. R. Simon for carrying out the auto-hemolysis tests. Moving boundary electrophoresis was kindly carried out by Dr. D. Rigas, Portland, Ore.

REFERENCES


Hemoglobin-Seattle (α₂Aβ₂β₂76 Glu): An Unstable Hemoglobin Causing Chronic Hemolytic Anemia

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