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Hemoglobin Cheverly: an Unstable Hemoglobin Associated with Chronic Mild Anemia

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Summary

The evaluation of a family with chronic mild anemia led to the identification of a new unstable hemoglobin (Hemoglobin Cheverly). Modest anemia and reticulocytosis, normal to slightly increased mean corpuscular volume (MCV), and normal mean corpuscular hemoglobin concentration (MCHC) were present in the affected family members. Electrophoresis of blood samples on cellulose acetate and on citrate agar revealed normal patterns. Globin chain analysis and isoelectric focusing data were also normal. After incubation for 3 h at 41°C, Heinz bodies were detected in 95–100% of erythrocytes from affected individuals. Positive heat and isopropanol tests confirmed the initial observation of the Heinz body preparation and indicated that an unstable hemoglobin was present. Structural analysis showed an amino acid substitution of Phe-Ser at position 45 (CD4) in the β chain. Hemoglobin Cheverly has a reduced affinity for oxygen and a reduced Bohr effect, properties that can be rationalized on the basis of the x-ray crystallographic structure of normal hemoglobin.

Despite structural and functional similarities between Hb Cheverly and Hb Hammersmith, β42 (CD1) Phe-Ser, the clinical manifestations of Hb Cheverly are mild in contrast to the severe disease observed with Hb Hammersmith. Reasons for the apparently silent clinical expression of Hb Cheverly are not known. We discuss the implications of unstable hemoglobins in the evaluation of chronic anemia in pediatric patients.

Abbreviations

CM, carboxymethyl
G-6-PD, glucose-6-phosphate dehydrogenase
MCHC, mean corpuscular hemoglobin concentration
MCV, mean corpuscular volume
6-PGD, 6-phosphogluconic acid dehydrogenase
PTH, phenylthiohydantoin

Elucidation of the etiology of chronic anemia in childhood may be difficult. Although iron deficiency and thalassemia are commonly encountered in the pediatric group, the pediatrician may be confronted with the patient who demonstrates chronic anemia, often with modest reticulocytosis, where the usual tests (*e.g.*, serum ferritin, quantitative electrophoresis for hemoglobin A_2 and F, erythrocyte indices) are unremarkable and where no chronic nonhematologic disease is identified.

In this communication we report on a family with chronic mild anemia that was unresponsive to hematinic therapy, and led to the identification of a new unstable hemoglobin (Hemoglobin Cheverly). The clinical and biochemical features of Hemoglobin Cheverly are described. We discuss the implications of unstable hemoglobins in the evaluation of chronic anemia in pediatric patients.

CASE REPORT

A, a white female child, was referred to the Johns Hopkins Hospital at the age of 7 years for evaluation of chronic mild anemia, which was refractory to iron therapy. She was the 3830 g product of an uncomplicated pregnancy and had no hyperbilirubinemia or anemia in the neonatal period. A low hemoglobin level (8.7 g/dl) was first detected at age 21 months. Examination of the peripheral blood smear disclosed slight hypochromia and anisocytosis. The patient received a therapeutic trial of multivitamins with iron, but the anemia persisted. Over the next 5 years, the patient received several courses of oral elemental iron therapy, without improvement in hemoglobin values despite documented normal serum iron levels. Her anemia was characterized by modest reduction in hemoglobin levels (range 8.5–9.5 g/dl) and slight reticulocytosis (5–8%). She had a urinary tract infection at age 4 years, which was treated with a sulfonamide without adverse effects. The patient's growth and development were entirely normal. Anemia of unknown etiology, similar to that observed in the proposita, was also found in her 4-year old male sibling and in her mother, whose anemia persisted despite trials of iron, folate, vitamin B12, and other hematinics. The father of the patient was hematologically normal.

Physical examination revealed a well-developed, well-nourished child in no distress. There was no frontal bossing or maxillary hyperplasia. Examination of lungs and cardiovascular system was unremarkable. There was no hepatosplenomegaly or skeletal anomalies. Neurologic examination was normal.

MATERIALS AND METHODS

Hematologic studies were performed with a Coulter Model S electronic counter and by standard methods (9). Osmotic fragility studies were done on both fresh and incubated blood samples according to the method of Dacie and Lewis (9). Levels of glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconic acid dehydrogenase (6-PGD) were assayed by a modification of the method of Glock and McLean as modified by Zinkham and Lenhard (33). Hexokinase activity was determined according to the method of Chapman *et al.* (6). Pyruvate kinase was assayed by the technique of Tanaka *et al.* (27).

Electrophoretic procedures for the detection of abnormal hemoglobins on cellulose acetate and on citrate agar, as well as analysis of globin chains at acid and alkaline pHs, have been described (20). Hemoglobin A_2 was quantitated by micro-chromatography (11), and the % of alkali-resistant hemoglobin was determined by the method of Singer *et al.* (23). The isopropanol and heat tests for unstable hemoglobin were performed as reported elsewhere (4, 18). Blood smears were examined for inclusions (Heinz bodies) according to the method of Simpson *et al.* (22).

Globin chains were isolated by the method of Clegg *et al.* (8) after heme had been removed by treatment with a mixture of

acid-acetone. After reduction with dithioerythritol, the chains were aminoethylated (17) and then digested with trypsin (24). The digest was analyzed by peptide fingerprinting at pH 6.4 (1) and by column chromatography in pyridine-acetate buffers (12). Rechromatography on Dowex 1 x 2 was done according to the method of Schroeder (21).

Peptides were hydrolyzed in 6 N HCl at 110°C for 24 h *in vacuo* and analyzed on a Beckman 121 amino acid analyzer (26). Sequence analysis was carried out on a Beckman 890C sequencer according to the programs (122974 and 121078) provided by the manufacturer, but with modifications to use 0.5 M quadrol. The PTH-derivatives were identified and quantitated by high performance liquid chromatography (25).

Oxygen equilibrium studies were carried out using whole blood at constant pH and P_{CO_2} (29). The oxygen equilibrium curves for pure Hb Cheverly were estimated by the following sequence of procedures. First, oxygen equilibrium curves for Hb A blood were simulated for conditions of pH, 2,3-diphosphoglycerate, and P_{CO_2} at which the Hb A/Hb Cheverly curves were measured (30). Second, the Hb A curves were subtracted from the Hb A/Hb Cheverly curves. The approximate fraction of Hb Cheverly was estimated to be 40% using a computer procedure described previously (31).

All enzymes were purchased from Worthington Biochemical Corp., and other chemicals were of analytical grade.

RESULTS

Hematologic data on the proband and her family are shown in Table 1. Modest anemia and reticulocytosis, normal to slightly increased MCV, and normal MCHC were present in the affected family members. Levels of erythrocyte-associated enzymes were normal. Results of osmotic fragility studies on unincubated blood samples were normal in all four persons examined; however, osmotic fragility curves of sterile blood samples incubated for 24 h at 37°C showed slightly increased hemolysis in the three anemic family members. Of interest was the finding that these blood samples had assumed a chocolate-brown color after sterile incubation, indicating the formation of methemoglobin. Heinz bodies were detected after incubation for 3 h at 41°C in 95–100% of the erythrocytes from affected individuals, compared to 0–10% in controls.

Electrophoresis of blood samples on cellulose acetate and on citrate agar revealed normal patterns. Globin chain analysis and isoelectric focusing data were also normal, as were the results for quantitation of Hb A_2 (2.9%) and Hb F (0.9%). The positive heat and isopropanol tests confirmed the initial observation of the Heinz bodies preparation and indicated that an unstable hemoglobin was present in the three family members who are anemic.

Further structural studies were done on hemolysates from the proband's mother. Globin was prepared by heat precipitation at 60°C. After removal of heme in cold acid-acetone the chains were separated by column chromatography. A disproportionate amount of chain migrating in the βA position was obtained as compared with chain migrating in the αA position. We have found that when unstable hemoglobins are precipitated by heat or isopropanol, the variant chain appears to be in excess after column chromatography on CM-cellulose (8).

The peptide fingerprint of the αA chain tryptic digest was normal. But there were two additional peptide spots on the fingerprint of the β-chain digest. These peptides were acidic, and separated between βTp-III and βTp-V. Amino acid analysis revealed that these two peptides have a composition identical to normal βTp-V, except that one of the three residues of phenylalanine has been replaced by serine (Table 2). The two abnormal peptides, although identical in amino acid composition, were located in different positions on the map because of the oxidation of methionine in one of the peptides. Normal βTp-V is also present, because βA chains were precipitated in the heat procedure. All other β chain peptides were accounted for.

The tryptic digest was also analyzed by column chromatography on ion-exchange resins. The abnormal βTp-V was isolated by rechromatography on Dowex 1 x 2 (Fig. 1). The composition, with a Phe-Ser substitution, was identical to that of the corresponding peptide isolated by peptide fingerprinting (Table 2). The normal βTp-V contains three residues of phenylalanine, at positions 41, 42, and 45. Automated sequence analysis of abnormal βTp-V shows that phenylalanine at position 45 is replaced by serine (Table 3).

Table 2. *Amino acid composition βTp-V Hb Cheverly*[1]

Amino acid	Expected	Found: Finger-printing	Found: Rechroma-tography
Lys	1	1.3	0.9
Asp	3	2.8	3.0
Thr	1	0.9	0.9
Ser	2	2.9	2.6
Glu	1	1.2	1.1
Pro	2	1.9	1.7
Gly	2	2.4	1.9
Ala	1	1.2	1.0
Val	1	0.8	0.9
Met	1	0.6	0.9
Leu	1	1.2	0.8
Phe	3	1.3	1.7

[1] All values are expressed as molar ratios. Samples were hydrolyzed at 110°C in 6 N HCl for 24 h *in vacuo*.

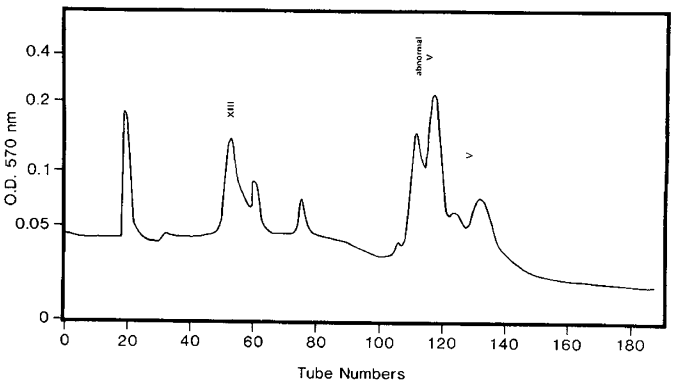

Fig. 1. Rechromatography of βTp-V on Dowex 1 × 2.

Table 1. *Hematologic data*

	A (proband)	B (mother)	C (brother)	D (father)
Hemoglobin (g/dl)	9.4	10.3	9.3	14.9
Hematocrit (%)	28.3	31.1	27.2	42.0
Red blood cell count (× 10^{-12}/liter)	3.45	3.47	3.28	4.74
Mean corpuscular volume (fl)	82	89	82	89
Mean corpuscular hemoglobin concentration (g/dl)	33.4	33.2	34.4	35.5
Reticulocytes (%)	3.4	3.6	4.2	1.8

The kinetics of the heat denaturation of Hb A/Hb Cheverly is shown in Figure 2. There is an initial short lag period, probably due to temperature equilibration, before the more rapid increase in the denaturation of hemoglobin occurs, as compared to a control. The shape of the curve is not linear, which is likely a reflection of the mixed hemolysate containing both the unstable variant and Hb A.

We have subjected these data to further computations, and the results are shown in Figure 3. We have assumed that the denaturation of the unstable hemoglobin (Hb Cheverly) is a linear process,

Table 3. *Sequence Hb Cheverly*

Cycle No.	Residue No.	Identification	nmoles
1	41	Phe	58.1
2	42	Phe	65.0
3	43	Glu	34.6
4	44	Ser	4.5
5	45	Ser	6.8
6	46	Gly	30.4
7	47	Asp	19.9
8	48	Leu	13.3
9	49	Ser	2.8
10	50	Thr	1.6

[1] All samples identified by high performance liquid chromatography. Initial sample was 150 nmoles.

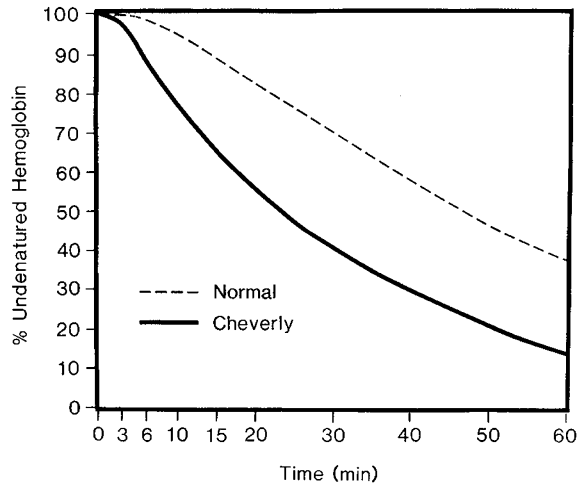

Fig. 2. Heat denaturation studies of a hemolysate of HbA/Hb Cheverly and HbA. Heat denaturation was carried out at 65°C in 0.2 M NaCl. Other experimental details can be found in Reference 18.

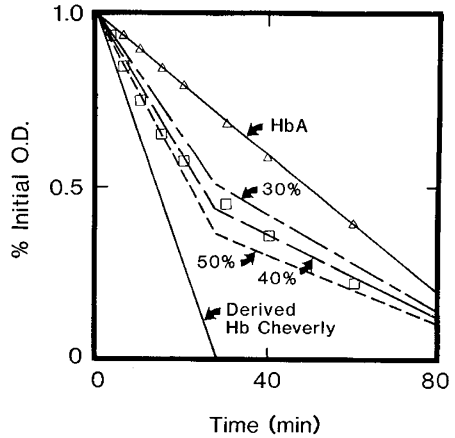

Fig. 3. Calculated values for the heat denaturation of Hb Cheverly. The results indicate the best fit to the experimental data is a 40–50% mixture of Hb Cheverly/HbA. See text for details.

and a theoretical curve has been constructed. Additional curves have been constructed based on the denaturation rate of Hb A and the derived rate for Hb Cheverly, but incorporating various concentrations of Hb A and Hb Cheverly. The results indicate that the best fit to the experimental data is a 40–50% mixture of Hb Cheverly to Hb A. This is consistent with the structural findings that Hb Cheverly is a β chain variant, and with the oxygen equilibrium studies.

Oxygen equilibrium studies with whole blood show that Hb Cheverly has a reduced affinity for oxygen as compared to Hb A (Fig. 4). The P_{50} value for Hb A containing blood, at pH 7.4, P_{CO_2}, 40 torr, is 28.7 torr. The value for Hb A/Hb Cheverly blood is 38.7 torr, under the same experimental conditions. The concentration of 2,3-diphosphoglycerate was within normal limits. Oxygen affinity measurements at different pHs indicate that the Bohr effect is approximately two-thirds of normal.

Although the possible contribution of methemoglobin (not measured) to the oxygen affinity determinations could not be estimated, extensive studies have shown that there is little or no increase in the concentration during measurement of the oxygen equilibrium curves of whole blood (29). In addition these experiments were done in the presence of catalase, which precludes the introduction of hydrogen peroxide into the cells.

If we assume that Hb Cheverly constitutes 50% of the hemoglobin, an oxygen equilibrium curve can be calculated for the pure variant by subtracting the contribution of Hb A from the observed oxygen equilibrium curve. These data are presented in Figure 5, where the open circles represent the calculated oxygen equilibrium curve of the pure variant and its fit to the Adair stepwise oxygen-

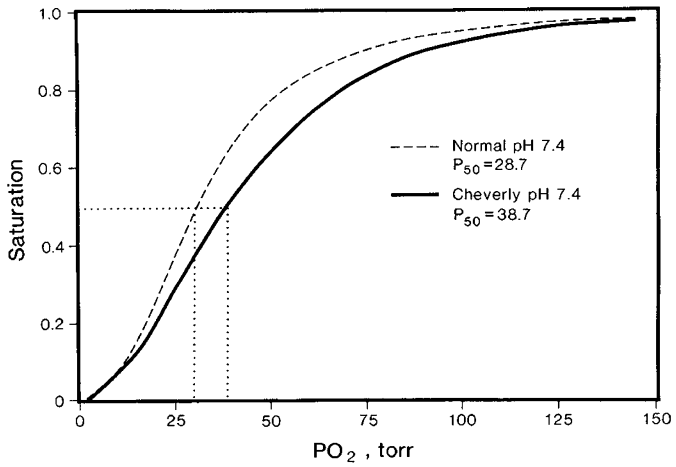

Fig. 4. Oxygen equilibrium curves of HbA and HbA/Hb Cheverly containing blood. Experimental details are given in Ref. 29.

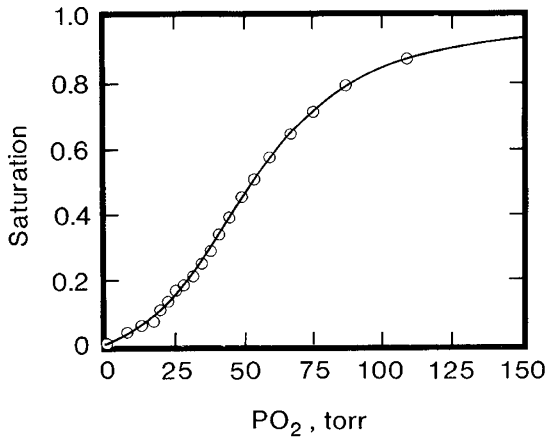

Fig. 5. Calculated oxygen equilibrium curve of Hb Cheverly and its fit to the Adair equation.

ation scheme. The partial pressure at 50% saturation for pure Hb Cheverly was determined to be 53.7 torr at pH 7.4, and the cooperativity was normal ($n = 2.62$).

DISCUSSION

Our account of the characterization of Hb Cheverly shows that the substitution of phenylalanine by serine at position 45 in the β chain leads to instability and reduced oxygen affinity of the variant. One mechanism for instability of the hemoglobin molecule is the perturbation of heme-contact sites, usually due to the substitution or deletion of single amino acids in the CD, E, F, FG, or H regions of the α and β globin chains. In Hb A, β45 (CD4) Phe is an external residue which is partially buried in the heme pocket (15, 16). It makes contact on the distal side (*i.e.*, on the side of the E helix) through the propionic acid of pyrrole III. It is close to β42 (CD1) Phe, which also makes contact with pyrrole III, and to the adjacent methene bridge. The substitution of Phe-Ser at (CD1) in Hb Hammersmith (10) and at (CD4) in Hb Cheverly gives rise to properties that are very similar. This substitution in both variants introduces a small residue with hydrophilic properties for a large nonpolar hydrophobic residue.

The observed instability of Hb Cheverly is most likely due to the disruption of the bond between (CD4) Phe and heme. In addition, this change could be communicated to the even more critical (CD1) Phe, where substitution to serine in Hb Hammersmith leads to an opening of the heme pocket. This opening provides access of water to the heme pocket and results in instability of the variant. The distal histidine (E7) that makes contact with heme through the propionic group of pyrrole III could also be affected by the Phe-Ser substitution in Hb Cheverly and could be a contributing factor to the observed instability.

The increased stabilization of the deoxy or T structure of Hb Cheverly to account for the reduced affinity for oxygen is not clear. The original explanation applied to Hb Hammersmith suggested that the substitution of serine for phenylalanine led to a more upright displacement of heme as compared to that in Hb A, where phenylalanine lies close to and is nearly parallel to heme. This displacement is considered to favor the deoxy conformation. A similar explanation could be invoked for Hb Cheverly in that the effect of this mutation might be communicated to (CD1) or that breaking the bond between (CD4) and heme might produce a similar effect, because the orientation of Phe (CD4) is very similar to that of Phe (CD1). An additional consideration is that Phe is replaced by a hydrophilic residue that might be able to make contact with another group and in this way increase the stability of the deoxy conformation; thus, the low oxygen affinity of Hb Cheverly may be mediated in some way through changes that occur in the heme pocket.

The observed Bohr effect is two-thirds of normal. Although the substitution at (CD4) does not directly involve a Bohr residue, there are indirect contacts. These principally involve secondary contacts through α141 (HC3) Arg, which is linked by a salt bridge to (NA1) Val of the other α-chain. The latter is a Bohr residue (14, 19). More difficult to explain is the reduced Bohr effect and the observed normal cooperativity of Hb Cheverly. These properties of hemoglobin are interrelated, and a decrease in the Bohr effect is generally associated with a decrease in cooperativity because both require a stable T conformation. Our experiments were done with blood containing a mixture of Hb A and Hb Cheverly; thus, the possible contribution of mixed hybrids or of methemoglobin (not measured) to the results obtained could not be determined. Although experiments with pure Hb Cheverly would provide an answer, these data are unobtainable because Hb A and Hb Cheverly cannot be separated.

Despite structural and functional similarities between Hb Cheverly and Hb Hammersmith, the clinical manifestations of Hb Cheverly are mild, in contrast to the severe disease observed with Hb Hammersmith (10). Our patient did not develop jaundice or exacerbation of anemia during febrile illnesses or after exposure to sulfonamides, situations that have been associated with increased hemolysis in subjects with moderately to severely unstable hemoglobins (5, 28, 32, 34).

Reasons for the apparently "silent" clinical expression of Hb Cheverly are not known. That other hemoglobin variants in the CD region of the β chain may be associated with mild symptoms is well recognized. Hemoglobin Louisville (Bucuresti), in which leucine replaces phenylalanine at residue 42 (CD1) of the β chain, represents a substitution by a hydrophobic amino acid residue, and therefore results in relatively minimal impairment of heme-globin interaction (2, 13). Interestingly, patients with Hb Louisville are slightly anemic and form Heinz bodies only after incubation with redox dyes, whereas patients with Hb Bucuresti demonstrate more striking reduction in hemoglobin levels and also exhibit Heinz body formation on initial staining with Nile blue dye. In Hemoglobin Okaloosa, in which arginine is substituted for leucine at residue 48 (CD7) in the β globin molecule, affected individuals are asymptomatic and demonstrate low normal hemoglobin values with modest reticulocytosis (7).

A common denominator in patients with these variants that are associated with mild symptoms is the observation that the magnitude of reduction in hemoglobin levels is more often related to the oxygen affinity of the hemoglobin variant than it is to the degree of hemolysis. For low affinity variants, oxygen delivery to the tissues is enhanced despite anemia, and there is less stimulus to erythropoiesis. Conversely, patients with unstable hemoglobins, which demonstrate high oxygen affinity, tend to have normal or near-normal hemoglobin levels (3).

The structural identification of an unstable hemoglobin is often the culmination of extensive and costly diagnostic efforts in the evaluation of a child with chronic anemia; however, a few selected laboratory screening procedures will indicate whether or not an unstable hemoglobin is responsible for reduction in hemoglobin levels and/or persistent reticulocytosis, and may be judiciously employed in most clinical centers to help to diagnose and to differentiate the etiology of anemia in pediatric patients.

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