Hemoglobin Brigham ($\alpha_2^A\beta_2^{100\ Pro \rightarrow Leu}$)

HEMOGLOBIN VARIANT ASSOCIATED WITH FAMILIAL ERYTHROCYTOSIS

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ABSTRACT Erythrocytosis associated with the presence of a hemoglobin with increased oxygen affinity has been reported for 10 hemoglobin variants, most of which demonstrate altered electrophoretic mobility. Several members of a family were found to have erythrocytosis, and both the whole blood and the hemoglobin exhibited increased oxygen affinity. Phosphate-free hemoglobin solutions had a normal Bohr effect and reactivity to 2,3-diphosphoglycerate. The electrophoretic properties of the hemoglobin were normal, but on peptide mapping of a tryptic digest of the isolated β-chains, a normal βT11 peptide and an abnormal βT11 with greater R_f were seen. Analysis of the abnormal peptide showed the substitution of leucine for the normal proline at β100 (helical residue G2).

The hemoglobin variant, designated Hb Brigham, serves to emphasize the necessity for detailed evaluation of the structure and function of hemoglobin in familial erythrocytosis even with electrophoretically "normal" hemoglobin.

INTRODUCTION

Familial erythrocytosis is likely to be related to the presence of an abnormal hemoglobin with increased affinity for oxygen. At least 10 different variants have been reported (1–12). With the exception of Hb Olympia (12), these abnormal hemoglobins can be separated from Hb A by electrophoresis or chromatography.

This report presents studies of a family with hereditary erythrocytosis in whom the whole blood and hemoglobin showed increased oxygen affinity. The electrophoretic and chromatographic behavior of the hemoglobin was identical to that of Hb A. An abnormal hemoglobin in which leucine replaced proline at residue β100 (G2) was ultimately found to account for about 50% of the circulating hemoglobin. This variant is designated Hb Brigham.

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METHODS

Laboratory data, including blood counts, reticulocyte counts, values for serum iron, and leukocyte alkaline phosphatase, were obtained on all available members of the four generations of the family. Only children of parents known to possess the abnormal hemoglobin were evaluated.

For determinations of partial pressure of oxygen at which hemoglobin is half saturated (P_{50}),[1] venous blood was equilibrated with oxygen at different partial pressures, and the proportion of oxyhemoglobin was measured spectrophotometrically with a CO-oximeter (Instrumentation Laboratory, Inc., Lexington, Mass.) (13). Curves were corrected to pH 7.4 by the use of the following Bohr factor: $\Delta \log P_{O_2}/\Delta pH = -0.48$. In addition, whole blood P_{50} determinations were made by the method of mixing described by Edwards and Martin (14). Spectrophotometric oxygen equilibria were determined at 20°C on phosphate-free hemolysates as described previously (15). Solutions contained 0.1 mM hemoglobin tetramer in 0.1 M total chloride and 0.05 M bis-Tris buffer at different pH values. 2,3-diphosphoglycerate (DPG) levels were determined by Schröter and von Heyden's modification of the Krimsky method (16); erythropoietin (ESF) assays were carried out on serum using the rat bioassay (17) and the red cell hemagglutination-inhibition test (18).

Red blood cell and plasma volumes were measured using standard radioisotope techniques, and routine pulmonary function studies were performed utilizing standard methods.

Hemoglobin electrophoresis was carried out on cellulose acetate and starch gel (Tris-EDTA-borate buffers, pH 8.6), on citrate-agar (pH 6.2) and by isoelectric focusing

[1] *Abbreviations used in this paper:* 2,3-DPG, 2,3-diphosphoglycerate; ESF, erythropoietin; P_{50}, partial pressure of O_2 at which hemoglobin is half saturated with O_2; PMB, *p*-hydroxymercuribenzoate.

on polyacrylamide gel (19). The hemoglobin was treated with *p*-hydroxymercuribenzoate (PMB) according to the methods of Geraci, Parkhurst, and Gibson (20). The mobility of the subunits was ascertained by starch gel electrophoresis.

Hemolysates were prepared by lysis of washed erythrocytes with distilled water and toluene. The methods used for structural studies were those of Clegg, Naughton, and Weatherall (21) with minor modifications. Globin, prepared from the hemolysate by acid-acetone precipitation at −20°C, was separated into α- and β-chains by column chromatography on carboxymethylcellulose using 8 M urea. Each chain was aminoethylated and then subjected to digestion with trypsin (Worthington TRTPCK, 1 mg/100 mg globin, Worthington Biochemical Corp., Freehold, N. J.).

For peptide mapping, 5 mg of the α- or β-chain peptides were subjected to electrophoresis for 2½ h at 2,000 V in a Varsol-cooled tank (Varsol Solvents, Humble Oil & Refining Co., Houston, Tex.) with pyridine-acetic acid-water (1:1:78) buffer at pH 4.7. Ascending chromatography was done in a butanol-acetic acid-water-pyridine (15:3:7:10) mixture. Peptide maps were stained for specific amino acids (22). For improved separation, electrophoresis was carried out for 5 h in the same buffer, followed by chromatography. Peptide maps were stained by dipping or spraying 0.02% ninhydrin in acetone buffered with 5% pyridine-acetic acid-water (21:1:224) pH 6.4. After elution in 6 N HCl containing 9 mg phenol/100 ml, peptides were hydrolyzed in vacuum for 16 h at 110°C. A Beckman 120 C amino acid analyzer was used for amino acid analysis (Beckman Instruments, Inc., Fullerton, Calif.).

Case report. Mr. A. G. was found to have erythrocytosis at the age of 47 in 1956 when he was evaluated for symptoms of headache, fatigue, and angina pectoris. He was treated at that time with radioactive ^{32}P as well as with anticoagulants for presumed polycythemia vera. During the next 5 yr phlebotomies were done every 3 mo, and a second course of ^{32}P and three courses of an alkylating agent were given. In 1961 the patient was referred to the Peter Bent Brigham Hospital where neither splenomegaly, leukocytosis, nor thrombocytosis were noted. In 1962 blood volume studies showed a red cell mass of 30.6 cm³/kg (normal 25.2–31.8), a plasma volume of 28.2 cm³/kg (normal 37–46), and a total blood volume of 59.2 cm³/kg. The diagnosis at that time was stress polycythemia.

In 1969 two daughters of the propositus were found to have hematocrit values of 55% and 53%, and the possibility of an hereditary hemoglobinopathy was raised. Simultaneously, one brother was evaluated for polycythemia at another institution and found to have an increased red cell mass with normal plasma volume. Subsequent evaluation of the propositus revealed that the hemoglobin electrophoresis was normal by both starch gel and cellulose acetate electrophoresis. The hemoglobin oxygen dissociation curve was shifted to the left on several determinations (Fig. 1). No further therapy has been instituted, and the present hemoglobin of 18 g/100 ml is well tolerated without symptoms. Blood volume studies in 1971 were as follows: red cell mass 36.4 ml/kg, plasma volume 36.8 ml/kg, and whole blood volume 5,631 ml (predicted normal whole blood volume was 4,636 ml).

RESULTS

Family studies. The family pedigree is shown in Fig. 2. Four generations were available for study with a total of 91 family members. 31 members were evaluated hematologically; increased oxygen affinity (low P_{50} values) of the whole blood was found in 10 (Table I). 10 additional members in whom P_{50} values were not obtained were suspected of having the abnormal hemoglobin for a variety of clinical reasons: nine of this putatively abnormal group had hemoglobins ranging from 16.0 to 18.9 g/100 ml, three of the group had elevated reticulocyte counts, and one additional patient, not included in Table I, was treated by phlebotomy for many years, although details of her hematologic status were not available. In the absence of P_{50} determinations it is not possible to make a definitive statement about these 10 subjects, but the clinical information suggested that they too possess Hb Brigham. In any case, the pattern of inheritance was consistent with that of an autosomal dominant (or codominant) trait.

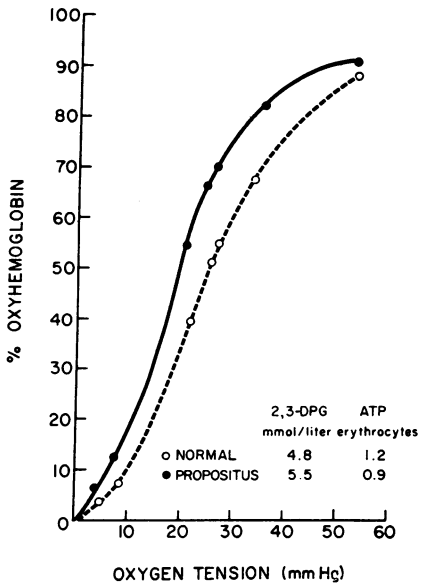

FIGURE 1 Whole blood oxygen dissociation curves, pH 7.40, 37°C: normal (○) and propositus (●). P_{50}: ○ = 25 mm Hg; ● = 19 mm Hg.

Clinical course. Evidence of the presence of the abnormal hemoglobin was detected in family members from age 2 to 81 yr, and the presence of an increased oxygen affinity of the blood did not appear to affect longevity.

No significant clinical symptoms appeared to be definitely related to the presence of the abnormal hemoglobin. One affected family member had fatigability and vertigo which were subjectively ameliorated by phlebotomy. 4 children with Hb Brigham had completely normal term development without complications, and five maternal carriers had 16 normal children without fetal wastage or maternal disability.

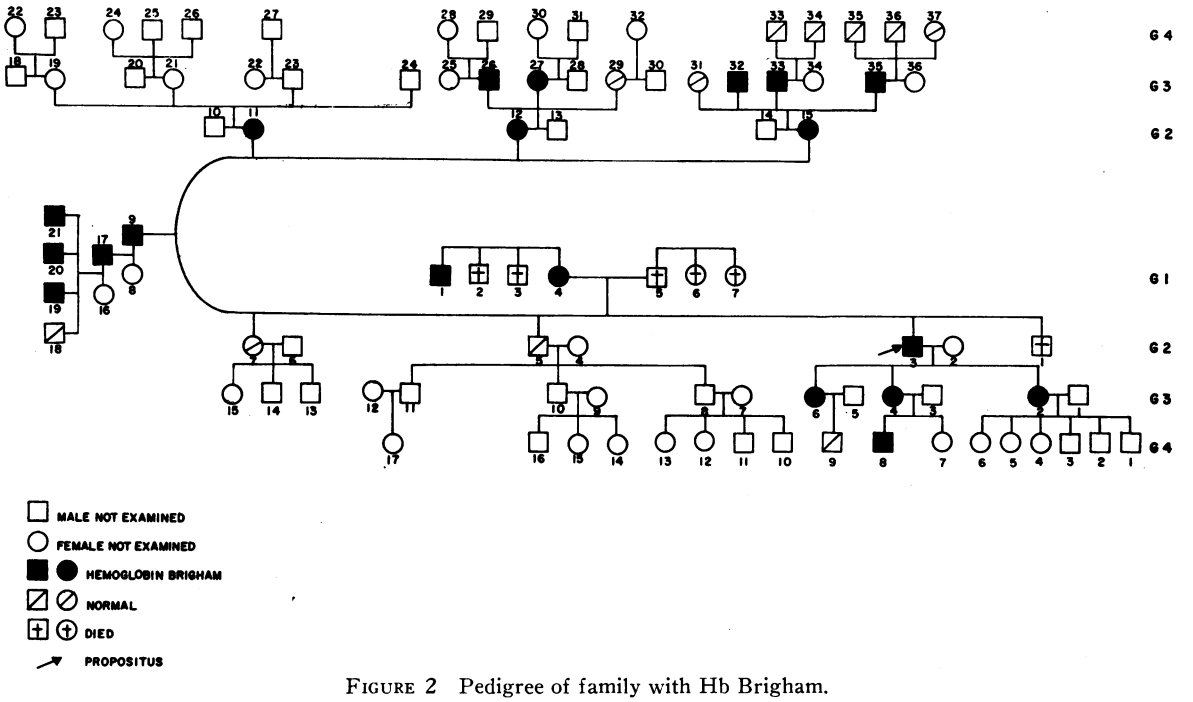

FIGURE 2 Pedigree of family with Hb Brigham.

Structural analysis of hemoglobin. Hemoglobin electrophoresis of blood of propositus on starch gels at alkaline pH and on citrate-agar at pH 6.2 revealed no abnormalities, nor did tests for heat stability and for Heinz body formation. In addition, no abnormal bands were detected on oxy and carboxy derivatives upon

TABLE I

Hematologic Data, P_{50} Determinations and 2,3-DPG Levels in Family Members with Hb Brigham

Generation member/number	Sex	Age	Hb	Hematocrit	Erythrocytes $\times 10^6$	Reticulocytes	P_{50} (normal 24.5–26.5)	2,3-DPG (normal 4.5–5.50)
			g/100g			%	*mm Hg*	*mm/liter cells*
Abnormal								
G2/3	M	60	20.0	58.8	6.1	3.0	21.7	5.50
G2/12	F	57	15.8	44.8	4.8	—	18.6	—
G2/15	F	54	16.8	49	5.0	4.2	21	—
G3/2	F	30	15.1	43	4.2	4.6	22	—
G3/6	F	26	15.4	46.9	5.2	—	18	4.90
G4/8	M	5	16.0	47.7	5.4	1.4	20	—
G3/35	M	28	17.9	52.3	5.2	2.2	19.3	4.42
G4/19	M	7	16.0	45.7	5.1	0.7	18.8	5.22
G4/20	M	6	16.1	46.8	5.1	1.1	18.0	4.81
G4/21	M	4	15.9	45.7	5.31	0.7	18.9	4.52
Probably abnormal								
G2/9	M	62	16.3	50	—	—	—	—
G1/4	M	81	16.8	47.4	5.2	4.2	—	—
G2/11	F	52	18.9	55	5.7	4.0	—	—
G3/4	F	32	17.4	51	5.6	1.0	—	5.00
G3/26	M	28	18.6	53.5	5.5	3.2	—	—
G3/27	F	26	16.0	46.3	4.9	1.2	—	—
G3/32	M	26	17.4	52.7	5.4	1.2	—	—
G3/33	M	20	16.8	51.1	4.9	0.4	—	—
G3/17	M	30	18.8	52.4	6.0	2.0	—	4.57

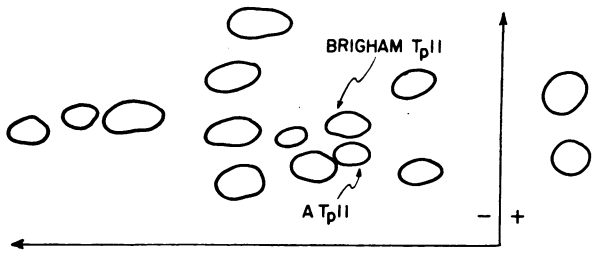

FIGURE 3 Tracing of a ninhydrin-stained fingerprint of aminoethylated and trypsin-digested β-chains from hemolysate containing Hb A and Brigham. Electrophoresis at 2,000 V for 3 h was followed by chromatography for 18 h.

gel electrofocusing. Furthermore, when deoxygenated hemoglobin was applied to the gels for electrofocusing under anaerobic conditions, no abnormality was detected. Starch gel electrophoresis of the PMB-treated hemoglobin reveals a pattern identical to that of similarly treated normal hemoglobin. Only one α- and one β-chain were isolated by urea column chromatography, and each was chromatographically identical with the α- and β-chains of normal hemoglobin. However, on peptide maps of the tryptic digest of the β-chain, an "extra" arginine and histidine positive spot was seen, electrophoretically identical to the normal βT_p11 but with a greater R_f (Fig. 3). Peptide maps in which electrophoresis was prolonged for 5-h contained the extra peptide relatively free of contaminating peptides: its amino acid composition was the same as that of normal βT_p11 except that proline was missing and two (rather than the expected one) residues of leucine were recovered (Table II). The sole proline in the normal β-peptide 11 is located at position 100 (G2) of the β-chain; therefore, the abnormal hemoglobin results from the replacement of the normal β100 (G2) proline by leucine.

Confirmatory evidence of the substitution was obtained by Dr. Vijay Sharma, who identified both proline and leucine as the fifth residue when a sample of the total peptide βT_p11 (normal plus abnormal) was subjected to automated Edman sequencing. As judged from the intensity of staining and by the amino acid recoveries of the normal and variant βT_p11, Hb Brigham accounted for about half the hemoglobin.

Functional studies of hemoglobin. An oxygen saturation curve on whole blood in the propositus is shown in Fig. 1. The left shift ($P_{50} = 19$ mm Hg) indicated a significant increase in oxygen affinity, a finding that was confirmed by oxygen equilibria performed on the phosphate-free hemolysate. The P_{50} values measured at different pH's on the hemolysate containing Hb Brigham and on a normal Hb A hemolysate indicate the oxygen

TABLE II
Amino Acid Composition of Abnormal βT_p11

Amino acid	Found	Hb A βT_p11
Histidine	1.1	1
Arginine	1.0	1
Aspartic acid	1.9	2
Glutamic acid	1.1	1
Proline	*0.0*	1
Valine	0.9	1
Leucine	*2.1*	1
Phenylalanine	0.9	1

Sequence of βT_p11

Residue no.	96	97	98	99	100	101	102	103	104
Hb A:	Leu	His	Val	Asp	*Pro*	Glu	Asn	Phe	Arg
Hb Brigham:	Leu	His	Val	Asp	*Leu*	Glu	Asn	Phe	Arg

affinity of the Brigham hemolysate[2] to be approximately 30% less than normal (Fig. 4). No difference was noted in the Bohr effect of the normal and Brigham hemolysates. Fig. 5 shows the oxygen equilibria of the Brigham hemolysate graphed according to the Hill equation: $y/1 - y = (P_{O_2}/P_{50})^n$.[3] The Hill plots were curvilinear with lower slope at values of y below 0.5 (compared to normal hemoglobin in which curvilinear plots do not develop until y is less than 0.1). The lower portion of the curve probably represented oxygenation of Hb Brigham while the upper portion re-

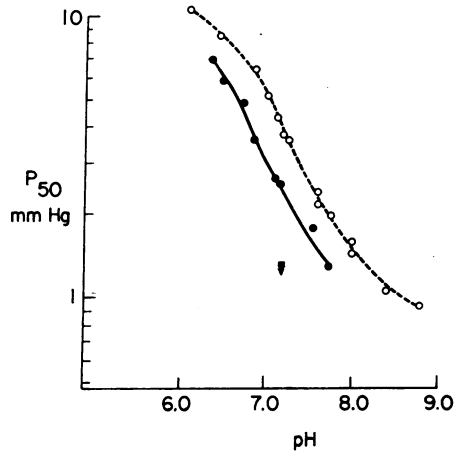

FIGURE 4 Alkaline Bohr effect of hemoglobin hemolysates: normal (○); Brigham (●); Kempsey (▼); Bethesda (■). (0.1 mM hemoglobin [tetramer] in 0.1 M chloride, 0.05 M bis-Tris buffer pH 6.0–8.8, 20°C). The Brigham, Kempsey, and Bethesda hemolysates contained approximately 50% hemoglobin A.

[2] The term "Brigham hemolysate" refers to the dual presence of Hb Brigham and Hb A in the hemolysate of affected individuals.

[3] y = fractional saturation of hemoglobin with oxygen. The coefficient superscript n is a measure of subunit cooperativity (heme-heme interaction).

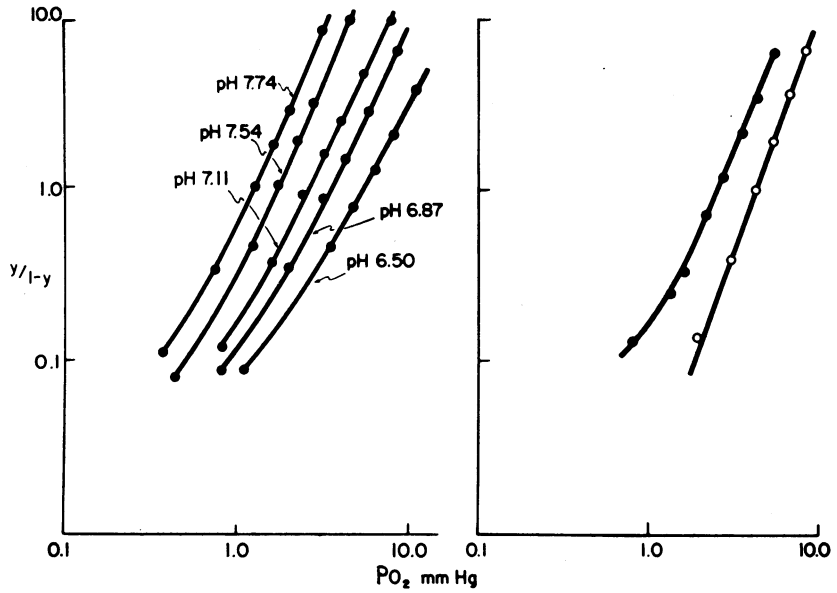

FIGURE 5 Left panel: Hill plot of oxygen dissociation curves of phosphate-free Brigham hemolysate (●). Same conditions as Fig. 4. Right panel: comparison of normal (○) and Brigham (●) phosphate-free hemolysates at pH 7.2.

flected oxygenation of Hb A contained within the hemolysate. The Bohr factor for $y = 0.25$ was 0.47, compared to 0.525 for $y = 0.75$. Thus on the basis of these data, Hb Brigham does not appear to have a Bohr effect significantly different from that of Hb A. This conclusion is supported by the inability to separate deoxyhemoglobins A and Brigham by gel electrofocusing.

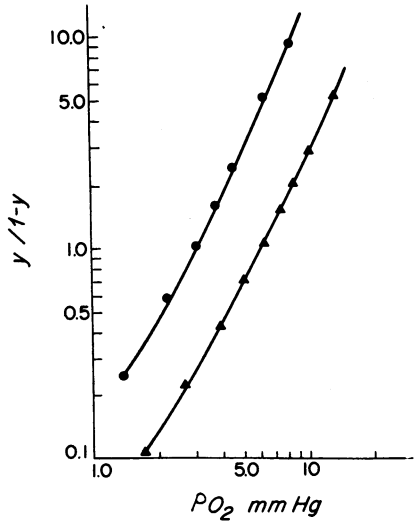

FIGURE 6 Hill plot showing the effect of 2,3-DPG on oxygenation of Brigham hemolysate: phosphate-free (●); 0.5 mM 2, 3-DPG (▲). Same conditions as Fig. 4 (pH 7.2).

The increase in isoelectric point of hemoglobin on deoxygenation is a direct reflection of the alkaline Bohr effect.

A uniform right shift of the Brigham hemolysate was observed in the presence of 2,3-DPG as shown in Fig. 6. The P_{50} of Brigham hemolysate increased from 2.9 mm Hg to 6.0 mm Hg by the addition of 0.5 mM 2,3-DPG. In comparison, the P_{50} of normal hemolysate went from 4.0 mm Hg to 8.0 mm Hg under identical conditions. Thus hemoglobin Brigham appears to have normal reactivity with 2,3-DPG.

The hemolysate of the propositus had a normal absorption spectrum in the visible region (400–700 nm). In particular the phosphate-free deoxy derivative showed an absorption pattern in the Soret region (400–460 nm) indistinguishable from that of Hb A. The cyanmethemoglobin derivatives of a normal hemolysate and that of the propositus both showed an OD_{540}/OD_{280} of 0.40, indicating a normal heme: globin ratio in Hb Brigham.

CO-combination and O_2-dissociation reactions were studied by stopped-flow method. The time-course of the reactions was monophasic and yielded rate constants similar to the corresponding rate constants for normal hemoglobin, indicating that the kinetic parameters of the abnormal component are not sufficiently differentiated from those of Hb A.[4] Definitive functional studies on Hb Brigham will not be possible unless it can be isolated free of Hb A.

[4] Sharma, V., and R. W. Noble. Unpublished data.

TABLE III

Stable Hemoglobin Variants with Increased Oxygen Affinity Associated with Familial Erythrocytosis

Reference no.	Hemoglobin	Substitution	Bohr effect	ESF	2, 3-DPG
α-variants					
1	Chesapeake	92 Arg → Leu	Normal	Normal (plasma)	Normal*
2	J Capetown	92 Arg → Gly	Normal	NR	NR
β-variants					
12	Olympia	20 Val → Met	NR	NR	NR
10, 11, 31	Malmö	97 His → Gln	Normal	NR	Normal (31)
3, 4	Yakima	99 Asp → His	Present	Normal (urine)	NR
5	Kempsey	99 Asp → Asn	Decreased‡	NR	Normal‡
8	Ypsilanti	99 Asp → Tyr§	NR	NR	NR
This report	*Brigham*	100 Pro → Leu	Probably Normal	Normal (serum)	Normal
6	Rainier	145 Tyr → Cys	Decreased	Normal (serum)	NR
9, 28, 29	Bethesda	145 Tyr → His	Decreased	High normal (urine)	Normal
7	Hiroshima	146 His → Asp	Decreased	NR	NR

NR, not reported.

* S. Charache. Unpublished data.

‡ H. F. Bunn. Unpublished data.

§ D. L. Rucknagel. Unpublished data.

2,3-DPG levels. Values for 2,3-DPG in eight patients ranged from 4.42 to 5.50 mmol/liter of cells. Analysis of multiple blood samples in some patients yielded consistently reproducible results. These values are essentially normal; no differences were noted between affected and unaffected family members.

ESF assay. Serum was analyzed for ESF by the rat bioassay method and the red cell hemagglutination inhibition test through the courtesy of Dr. Robert Lange, and found to be normal. Response to hypoxic stress or phlebotomy was not determined.

DISCUSSION

Familial erythrocytosis as a clinical manifestation of a structurally abnormal hemoglobin, Hb Chesapeake ($\alpha_2^{92\ Leu}\beta_2^A$), was first described in 1966 by Charache, Weatherall, and Clegg (1). Since 1966, 11 structural variants of hemoglobin (including Hb Brigham) have been reported in which a hemoglobin with a high oxygen affinity is accompanied by erythrocytosis (Table III). (A number of the unstable hemoglobins also have a high oxygen affinity, but they are generally classified as unstable rather than high affinity variants in accordance with the clinical manifestations of hemolysis rather than erythrocytosis.) Only two of the stable hemoglobins that have led to erythrocytosis, Hb Chesapeake and Hb J Capetown, are α-chain variants. Hb Chesapeake has a greater oxygen affinity and is accompanied by a more consistent and significant erythrocytosis than is Hb J Capetown. Both Hb Chesapeake and Hb J Capetown contain substitutions (leucine and glycine, respectively) for the normal α92 arginine at the $\alpha_1\beta_2$ area of interchain contact. The molecular mechanisms by which these substitutions lead to the increased ligand affinity are not established, although it is probable that the liganded (high affinity) form is somehow favored in Hb Chesapeake (23).

Of the stable β-chain variants with increased oxygen affinity, three (Yakima, Kempsey, and Ypsilanti), contain substitutions at β99 (helical residue G1) where the normal aspartic acid is replaced by histidine, asparagine, or tyrosine, respectively. This area of the $\alpha_1\beta_2$ contact undergoes a "click" from one dovetailed position to another on oxygenation (24), and the hydrogen bond between aspartate G1 β2 and tyrosine C7 α1 in deoxyhemoglobin is broken in the oxy form, in which asparagine G4 β2 is hydrogen-bonded to aspartate G1 α1. The replacement of β-aspartate G1 would thus promote instability of the quaternary form of the deoxyhemoglobin variant.

According to Bolton and Perutz (24), the normal proline residue at β G2 (β100) (which is replaced by leucine in Hb Brigham) is part of the $\alpha_1\beta_2$ interchain contact in deoxyhemoglobin in which G2 β2 is within

4 Å of C3 α1 threonine. In the liganded form, however, G2 does not participate in an interchain contact. The available information does not permit formulation of the mechanism of the increased ligand affinity in Hb Brigham. Apparently, the substitution of leucine for proline at G2 β alters the dovetail contact area which has notable structural changes during reactions with oxygen. Because it is larger than proline, leucine might impair the participation of the neighboring C1 (β99) aspartate in the hydrogen bond of deoxyhemoglobin.

Hb G Georgia, in which leucine replaces the normal proline at α95 (G2), is the α-chain counterpart of Hb Brigham (25).[5] Hb G Georgia accounts for about one-fourth of the total hemoglobin in these patients, and despite an increased ligand affinity, is apparently not accompanied by erythrocytosis. Its cooperativity is decreased, and the Bohr effect may be slightly reduced. Unliganded Hb G Georgia is tetrameric, but the oxy form dissociates extensively to dimers. Detailed studies of the properties of Hb Brigham must await the development of techniques for separating the variant from the normal hemoglobin, but the relationships of G2α in the $\alpha_1\beta_2$ contact obviously differ from those of G2β, in that G2α forms a nonpolar contact with tryptophan C3β in both the liganded and unliganded forms. Furthermore, Hb Georgia, identified by electrophoresis, is more positively charged than Hb A, presumably as a result of conformational change induced by the amino acid substitution.

The pathophysiologic effect of the structural alteration in hemoglobins with a high affinity for oxygen is to create a state of relative hypoxia and a consequent increase in ESF production. ESF levels in previous reports (1, 6, 28), as well as in Hb Brigham patients, have been uniformly normal (Table III). The explanation for this apparent paradox is that the regulation of red blood cell production is reset at erythremic levels to provide the same tissue oxygenation, but with a hypoxic insult, such as phlebotomy, ESF levels will increase (6, 28).

2,3-DPG is an integral part of the molecular mechanism of oxygenation of hemoglobin. In the anemic state, increased 2,3-DPG levels effect a shift of oxygen dissociation curve to the right, facilitating release of oxygen to the tissues. An inverse relationship of 2,3-DPG to erythrocyte mass obtains, in that in primary erythrocytosis (polycythemia vera) reduced levels of 2,3-DPG are seen (30). In contrast, Hb Brigham, as well as four previously reported polycythemic hemoglobins (Table III), are associated with normal levels of 2,3-DPG.

The occurrence of Hb Olympia and Hb Brigham as electrophoretically silent hemoglobin variants, in which the detection of familial erythrocytosis led to subsequent intensive study of hemoglobin structure and function, serves to emphasize the limitations of routinely available methods for detecting hemoglobin variants. From considerations of possible single-step mutations of the genetic code, it appears that in only about one-third of the variants will the substituting amino acid differ in charge from the amino acid it replaces, and hence have a distinctive electrophoretic mobility. Heat stability testing has resulted in the detection of some of these electrophoretically silent variants in hemolytic states; indeed, in the unstable hemoglobins a variant Hb containing the replacement of an amino acid by one of like charge may be identified with charge-dependent techniques, because of the increased positive charge induced by heme loss, or because of alterations in the ionization of other amino acids consequent on altered configuration of the protein. However, for unexplained erythrocytosis unaccompanied by alterations of the electrophoretic properties of the hemoglobin, the only available screening method is the determination of oxygen affinity of the whole blood (P_{50}), followed by detailed studies of hemoglobin structure. The observation that Hb Bethesda with high oxygen affinity apparently occurred as a spontaneous mutation (29) emphasizes the fact that the erythrocytosis may on occasion not be familial.

[5] In addition, two α-chain variants have other amino acid substitutions at this site: Rampa ($\alpha_2^{95\ Ser}\beta_2$) (25, 26) and Denmark Hill ($\alpha_2^{95\ Ala}\beta_2$) (27). It is of interest that both these variants have increased oxygen affinity, but affected individuals do not have erythrocytosis.

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