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New ultra-micro high-performance liquid chromatographic method for determining the γ chain composition of hemoglobin F in normal adults

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ABSTRACT

The difficulty in isolating the minute quantity of Hb F (< 1%) present in the red blood cells of normal adults greatly complicates the determination of its γ chain composition. We have developed a rapid ultra-micro high-performance liquid chromatographic (HPLC) method and used it to analyze the γ chain composition of Hb F in 47 adults with Hb F levels between 0.1–3.4%. The method involves the isolation of Hb F from as little as 50 μl of whole blood on an analytical size cation-exchange HPLC column, followed by concentration in a Centricon micro concentrator unit and by reversed-phase HPLC analysis. The entire procedure can be completed in one day and 3–4 analyses can be made simultaneously. We reanalyzed the blood samples from 22 subjects with known β-globin gene cluster haplotypes, and confirmed the association of high, low, and very low $^{G}\gamma$ levels with haplotypes A, B, and C, respectively. Also included are the results of DNA sequence analyses of the $^{G}\gamma$ and β promoters, and of the locus-control-region hypersensitive site-2 (LCR-HS-2) of the β-globin gene cluster in five subjects homozygous for haplotypes A, B or C; the data obtained failed to provide a satisfactory explanation for all the variations in the $^{G}\gamma$ levels that have been observed.

INTRODUCTION

The heterogeneity of the γ chain of human fetal hemoglobin (Hb F) was discovered in 1968 [1] when it was observed that position 136 of the γ chain can be occupied either by a glycine residue (in the $^{G}\gamma$ chain) or by an alanine residue (in the $^{A}\gamma$ chain). Two genes which control the production of these γ-globin chains are part of the β-globin gene cluster, located on the short arm of chromosome no. 11 where they are found in a 5′-ε-$^{G}\gamma$-$^{A}\gamma$-ψβ-δ-β-3′ gene arrangement [2,3]. In addition to the perinatal switch from fetal (γ) to adult (β) globin synthesis, there is a parallel change in the levels of the two types of γ chain, from high $^{G}\gamma$ (70%) in the newborn to high $^{A}\gamma$ (60%) in the adult [4,5]. However, some adults continue to have high $^{G}\gamma$ values in the trace (< 1%) amount of fetal Hb found in their red blood cells. Nearly all these individuals have a C → T mutation at −158 of the $^{G}\gamma$ promoter, detectable with the restriction enzyme Xmn I [6].

Several years ago we analyzed the γ chain composition in normal adults with Hb F levels ranging from 0.1–2.5%, and also determined the β-globin gene cluster haplotypes. Those with subhaplotype A in the 5′ β-globin gene cluster region ([− + + − + +], restriction sites: Hinc II 5′ to ε, Xmn I 5′ to $^{G}\gamma$, Hind III in $^{G}\gamma$ and $^{A}\gamma$, and Hinc II in ψβ and 3′ to it) had high $^{G}\gamma$ levels (60–70%), while those with subhaplotype B [− − − − − +] had low $^{G}\gamma$ (25–30%), and those with subhaplotype C [+ − − − − −]

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had very low $^{G}\gamma$ (10–15%) levels [7]. This observation led to further attempts to analyze the minute quantity of Hb F that is present in blood from normal adults. The difficulty of isolating Hb F from blood samples of persons with Hb F levels below 1% greatly complicates the accurate determination of its γ chain composition. Here we introduce a new ultra-micro HPLC method and present the results of its application to the analysis of the γ chain composition of Hb F from 47 normal adults with Hb F levels between 0.1–3.4%. Twenty-one of these 47 persons had known haplotypes and the newest data confirmed the association of high, low, and very low $^{G}\gamma$ levels with subhaplotypes A, B, and C. Additional studies concern an evaluation of a possible relationship between different levels of $^{G}\gamma$ in the Hb F of three adults with a homozygosity for one of these haplotypes and DNA sequence variations in the $^{G}\gamma$ and β promoter regions and in the HS-2 segment of the LCR which is located 5′ to the ε gene [8].

EXPERIMENTAL

Blood samples from 47 adults with normal hematology and normal Hb A were analyzed. These included 22 previously studied subjects with known haplotypes [7,9]. Samples were collected in capillary micro hematocrit tubes or a small volume of 2 to 3 ml of blood was collected in a vacutainer tube with EDTA as anti-coagulant; this material was transported in ice to the laboratory, and next transferred into a capillary micro hematocrit tube. Routine hematological data were obtained with an automated cell counter (Sysmex K-1000, TOA Electronics, Kobe, Japan), and all samples were analyzed by isoelectrofocusing (IEF) [10]. Hb F values were determined by cation-exchange HPLC [11,12] and by an alkali denaturation procedure [13], whereas Hb A_2 was quantified by cation-exchange HPLC and DEAE-cellulose micro chromatography [14].

In this study we used an analytical size HPLC column (polyCAT-A; 200 mm × 4.6 mm I.D.; 5 μm particle size; Poly LC, Columbia, MD, USA) [15] for the isolation of Hb F. The blood in the hematocrit tube (*ca.* 50 to 60 μl) was transferred to an eppendorf tube and washed with 1.5 ml of 0.9% saline solution, whereafter the supernatant was removed with a Pasteur pipet. About 500 μl of deionized water was added to the red cell pellet. The content was vortex-mixed for *ca.* 2 to 3 min, and next centrifuged for 5 min at 13 000 *g* in a micro centrifuge. The concentration of the Hb in the hemolysate was measured spectrophotometrically and 0.5 to 1.5 mg of Hb was applied to an HPLC column.

A two-buffer system was used as a mobile phase. Developer A contained 35 m*M* Bis-Tris, 3 m*M* ammonium acetate, 1.5 m*M* KCN, pH 6.47, and developer B contained 35 m*M* Bis-Tris, 16.85 m*M* ammonium acetate, 225 m*M* sodium acetate, 1.5 m*M* KCN, pH 7.0. A gradient between these two solutions was developed for 60 min from 10% B to 50% B (90% A to 50% A), and for 30 min from 50% B to 90% B (50% A to 10% A). The polyCAT-A column was purged for 5 min with 100% B, and reequilibrated for 10 min with 10% B (90% A) prior to the next analysis. The absorbance of the effluent was continuously recorded at 415 nm with a Waters Data Module, Model 745 (Waters, Milford, MA, USA). The chart attenuation of the recorder was set to a less sensitive program (attenuation >128). Fig. 1 (top) provides an example showing the almost complete separation of Hb F and Hb A_{1c}. As the entire procedure requires only 100 min, it is possible to run 3 to 5 analyses in a working day. The Hb F together with a small amount of Hb A_1 was collected and concentrated in a Centricon micro concentrator unit (molecular mass cut-off 10 000; Amicon, Beverley, MS, USA) by centrifugation for 1 h at 2500 *g* to a volume of 50 to 150 μl (approximately 2 to 5 μg of Hb). This material was next injected onto a reversed-phase HPLC column (Vydac C_4; 250 mm × 4.6 mm I.D., large-pore size 330 Å, Vydac, Hesperia, CA, USA) for the determination of the globin chain composition.

We used a slightly modified reversed-phase HPLC procedure [16–18] in the present study. The two developers contained 60% ACN (acetonitrile), 0.1% TFA (trifluoroacetic acid), and

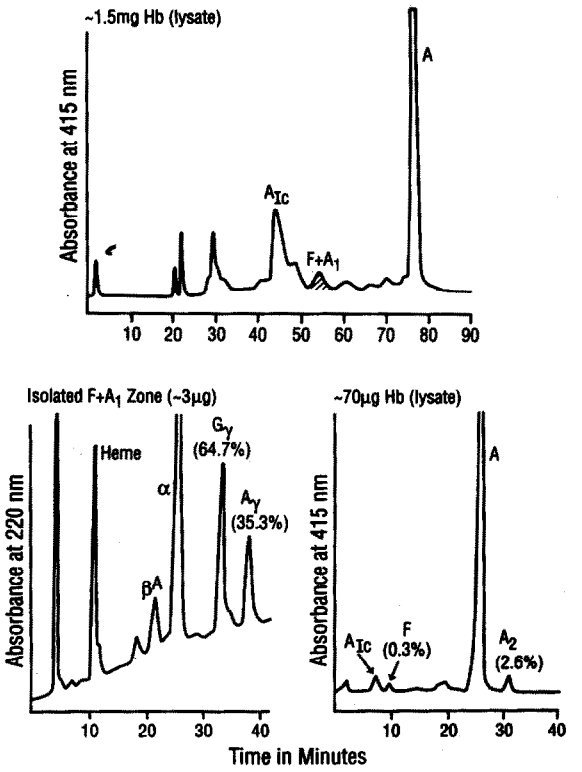

Fig. 1. Quantitation of Hb F of the $^G\gamma$ and $^A\gamma$ levels in *ca.* 50 to 60 µl blood collected from a normal adult in a hematocrit tube. Top: Isolation of an (impure) Hb F on a 200 mm × 4.6 mm I.D. polyCAT column. Bottom (left): Globin chain separation by reversed-phase HPLC; the concentrated F + A_1 zone from the chromatogram shown above was applied to a Vydac C_4 column. The identification of each zone is based on its position in the chromatogram. Bottom (right): Quantitation of Hb F on a polyCAT-A (200 mm × 4.6 mm I.D.) column. Past experience with samples containing slightly elevated levels of Hb F has indicated that the Hb F zone in this type of chromatography with a specific gradient system is virtually free of contaminating Hb A_1 components [11].

40% deionized water (developer A) or 20% ACN, 0.1% TFA, 80% deionized water (developer B). A linear gradient was used between the two developers. The chromatogram was completed in 50 min by using 48% A to 60% A (52% B to 40% B); the column was purged for 5 min with 100% A and reequilibrated for 5 min with 48% A (52% B). The effluent was monitored at 220 nm. The chart attenuation of the recorder was set to a highly sensitive (attenuation = 32–64) program. The integration was horizontal forward for all peaks (a base line correction is made automatically by the integrator). Fig. 1 (bottom left) illustrates the separation that was obtained. The quantitation of Hb F in the original sample was by cation-exchange HPLC as described before [11] (Fig. 1, bottom right).

Hb F was also isolated by DEAE-cellulose chromatography [14] and on a preparative-size cation-exchange HPLC column (polyCAT-A; 250 mm × 21 mm I.D., 12 µm particle size; Poly LC) as described before [11,18]. Its γ chain composition was determined by reversed-phase HPLC [16]. The comparison between the three methods provided information about the validity of the new method.

DNA from five persons with specific haplotypes was isolated with the method of Poncz *et al.* [19]. Sequencing of amplified segments of DNA containing the LCR-HS-2, the $^G\gamma$ promoter, and the β promoter regions of the β-globin gene cluster followed techniques used in earlier studies [20,21].

RESULTS

Blood samples were collected from 47 adult males and females with normal hematology, normal Hb A, Hb A_2 levels between 2–3%, and with Hb F values varying between 0.1 and 3.5%. Twelve of the 22 persons who had been studied before [7] were homozygous for haplotype A, four for haplotype B, one for haplotype C; three subjects had haplotypes A and C, and two had haplotypes B and C. The new HPLC procedure described here allowed a determination of the γ chain composition of the isolated Hb F in 41 of the 47 subjects. The data confirmed the previously observed association of high, low, and very low $^G\gamma$ levels with haplotypes A, B, and C, respectively (Fig. 2). The $^G\gamma$ levels varied between 60 and 86% (mean value 68.2%) for those with haplotype AA, between 20 and 44.3% (mean value 32.5%) for those with haplotype BB, and 12% for the one individual with haplotype CC. The Hb F levels varied between 0.1 and 3.5% (mean value 0.65%) in the individuals with haplotype AA, between 0.1 and 0.2% (mean value 0.15%) in the individuals with haplotype BB, and was 0.1% in the one individual with haplotype CC (Fig. 2).

TABLE I

HB AND SEQUENCE DATA FOR FIVE ADULT FEMALES WITH A HOMOZYGOSITY FOR SPECIFIC HAPLOTYPES

(values in %).

Case	Race	Haplotype[a]	F_{AD}		F_{Chr}	$^{G}\gamma$		LCR-HS-2			$^{G}\gamma$ Promoter			β Promoter	
			1986	1992	1992	1986	1992	−10924	−10906	AT repeat	−369	−309	−158	−551	AT/T
Reference sequence (from ref. 28)								TT	AA	10TA2CA2TACG11TA	CC	AA	CC	TT	7/7
C.B.	Caucasian	AA	0.9	1.0	0.1	58.1	66.0	TT	AA	9TA2CA2TACG10TA	CC	AA	TT	TT	7/7
										9TA2CA2TACG10TA					7/7
Sh.B.	Caucasian	AA	–	0.5	0.1	–	60.0	TT	AA	9TA2CA2TACG10TA	CC	AA	TT	CC	7/7
										9TA2CA2TACG10TA					3/11
B.W.	Black	BB	1.0	0.5	0.1	33.0	33.3	TG	AG	n.d.	CG	GG	CC	TC	7/7
															9/5
D.C.	Black	BB	1.6	0.5	0.2	26.5	20.0	TT	AA	9TA2CA2TACG10TA	GG	GG	CC	TC	7/7
										10TA2CA2TACG9TA					8/6
L.L.	Caucasian	CC	1.0	0.6	<0.1	12.0	9.3	TT	AA	8TA2CA2TACG11TA	CC	AA	CC	TC	7/7
										9TA2CA2TACG10TA					9/5

[a] A = [− + + − + + + + + +]; B = [− − − − − + + + + +]; C = [+ − − − − − + + + −]. Haplotype A corresponds to the β^S haplotype no. 3, haplotype B to β^S haplotype no. 19, and haplotype C to β^S or β^A haplotype no. 3 (= three minus the C → T mutation at −158 5′ to $^{G}\gamma$) (for references see 20, 21, and 24). n.d. = not determined.

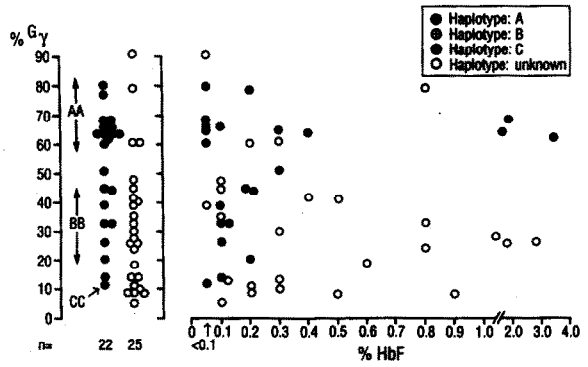

Fig. 2. The Hb F and $^{G}\gamma$ levels in 47 adults; 22 with known and 25 with unknown haplotypes.

In order to evaluate possible variations in $^{G}\gamma$ and $^{A}\gamma$ levels due to methodology, Hb F was isolated on a large HPLC column (250 mm × 21 mm I.D.) (28 subjects; 25 of these had a Hb F level of < 1%) and by DEAE-cellulose chromatography (22 cases) from the same set of blood samples.

Fig. 3 shows the correlation between the $^{G}\gamma$ levels in Hb F isolated with the different procedures, while Fig. 4 shows the relationship between the levels of Hb F in the original samples and the percentages of γ chains in the isolated Hb F + Hb A_1 fractions. The data indicate that the purity of the isolated material [as $\gamma/(\gamma + \beta)$] obtained with the ultra-micro HPLC method is considerably higher than that with the preparative HPLC column, and much better than that with the DEAE-cellulose chromatography, particularly in the lower Hb F range.

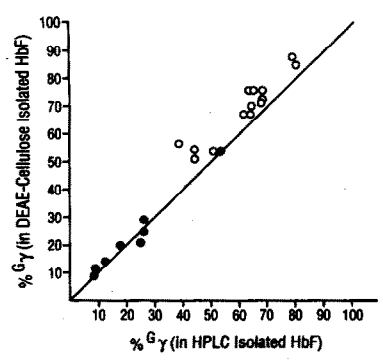

Fig. 3. Correlation between the $^{G}\gamma$ levels in Hb F isolated by DEAE-cellulose chromatography, and by (●) HPLC methodology (250 mm × 21 mm I.D.) and (○) HPLC methodology (200 mm × 4.6 mm I.D.) using the same set of samples.

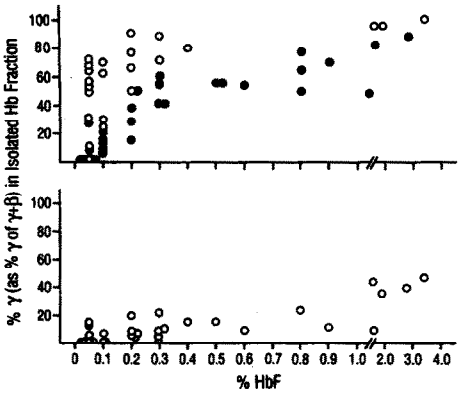

Fig. 4. Yield as % $\gamma/(\gamma + \beta)$ in isolated Hb F *versus* the Hb F levels in the original samples. Two different methods were used for the isolation of Hb F: (top) cation-exchange HPLC with column dimensions of (○) 200 × 4.6 mm I.D. and (●) 250 × 21 mm I.D.; (bottom) DEAE-cellulose chromatography.

The DNA sequence analyses were limited to five subjects, namely two Caucasians with haplotype AA, two Blacks with haplotype BB, and one Caucasian with haplotype CC. Sequence variations (in relation to the reference sequence) observed in the $^{G}\gamma$-globin gene promoter (at positions −369, −309, −158), in the LCR-HS-2 (at positions −10924, −10906; the repetitive purine/pyrimidine region between −10623 to −10670), and in the β-globin gene promoter (at position −551; number of AT/T repeats) of the DNA samples of the persons with these haplotypes are listed in Table I.

DISCUSSION

The HPLC method with the cation-exchange polyCAT-A column was introduced by Bissé and Wieland [12] and is used in our laboratory for the separation and quantitation of numerous normal and abnormal Hbs [11]. Here we have described a modification of this method applied to the isolation of low levels of Hb F (0.1–3.4%) found in normal adults. The method uses an analytical sized polyCAT-A column (200 mm × 4.6 mm

I.D., 5 μm particle size), which provides a larger surface to interact with Hb molecules when compared to the preparative column with the 12-μm particle size. The method requires minute quantities of whole blood (50–60 μl), which can be collected in a capillary micro hematocrit tube. As shown in Figs. 3 and 4, accurate quantitation of γ chains can be achieved for adults with Hb F levels less than 1%. The sensitivity of this method is superior to that of DEAE-cellulose chromatography (Fig. 4) and of methods which use alkali denaturation for the enrichment of Hb F in the Hb sample [22,23]. Although Enoki *et al.* [23] have obtained a reproducible Hb F enrichment with a modification of the existing alkali denaturation procedure, such a method is cumbersome, time-consuming, and may result in denaturation and consequent alteration of the chromatographic properties of the γ chains.

Analysis of the blood samples from previously studied subjects [7,9] confirmed the association of high (60–70%), low (25–30%), and very low (10–15%) $^{G}\gamma$ levels with haplotypes A, B, and C, respectively. The nucleotides in the $^{G}\gamma$ promoter at positions −369, −309, −158, in the LCR-HS-2 at positions −10924, −10906, and at the repetitive purine/pyrimidine region between positions −10623 to −10570 of the chromosome with haplotype A were identical to those found for β^{S} chromosomes with haplotype no. 3 (Senegal), which is associated with high Hb F and high $^{G}\gamma$ levels in sickle cell anemia (SS) patients [20,21]. Sequence variations observed for the two subjects with haplotype B were consistent with a compound heterozygosity for haplotype no. 19 (Benin) and an atypical haplotype no. 19A in one (B.W.), and homozygosity for the atypical haplotype no. 19B in the other (D.C.). This atypical haplotype, which likely results from a crossover between chromosomes with haplotypes no. 3 (Senegal) and no. 19 (Benin), and places the LCR-HS-2 sequence of haplotype no. 3 in juxtaposition to the γ-globin genes of haplotype no. 19, has been described in SS patients and in patients with β-thalassemia (β-thal) with high Hb F [21,24]. Subject L.L. who is homozygous for haplotype C (very low $^{G}\gamma$ levels) had the same nucleotides at the various positions as observed for subjects C.B. and Sh. B. with a homozygosity for haplotype A except for a C at position −158 to $^{G}\gamma$, while one chromosome also had a variation in the AT repeat of the LCR-HS-2 (Table I). Although the mechanism(s) responsible for the difference between low (haplotype B) and very low (haplotype C) $^{G}\gamma$ levels is (are) not readily explained by the DNA sequence variations observed in these regions, our data again indicate the importance of the C → T mutation at position −158 of the $^{G}\gamma$ promoter for the level of $^{G}\gamma$ even in normal persons.

The polymorphism around the −530 region of the β-globin involving the AT/T repeat which has been postulated to affect the β-globin gene expression and the level of the Hb F, particularly in anemic patients because of the variations in the binding of the nucleoprotein BP-1 [25–27] showed no direct correlation with the Hb F or $^{G}\gamma$ levels in normal adults. The same was recently observed for over 40 patients with β-thal major and a variety of β-thal mutations [28]. Thus, no obvious explanation for the variation in $^{G}\gamma$ values of the Hb F from normal adults with either haplotypes BB or CC has yet been obtained.

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