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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODOLOGY TO THE ANALYSIS OF HEMOGLOBINS SYNTHESIZED IN ERYTHROID PROGENITOR CELLS

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SUMMARY

High-performance liquid chromatography (HPLC) has been successfully used in the quantitation of the relatively minute amounts of hemoglobin types recovered from in vitro cultures of hemoglobin-synthesizing erythroid progenitor (BFU-E) cells. This reversed-phase HPLC method uses the Vydac C_4 column and water–acetonitrile–trifluoroacetic acid as mobile phases; it has been applied to the study of fetal hemoglobin synthesis patterns in ten homozygous sickle cell anemia patients and a similar number of their heterozygous relatives along with a few normal control subjects. A significant increase in the total γ chain level was observed in the BFU-E lysate samples corresponding to the whole blood lysates of all the patients and their heterozygous relatives, except in one patient with the β^S haplotype Mor. On the other hand, the relative level of the $^G\gamma$ chains appeared to be decreased in the BFU-E lysate samples of all except the individuals carrying the Mor haplotype, where it is reversed. The method has considerable advantages over other chromatographic and electrophoretic procedures; it is extremely sensitive and allows quantitation of all different globin chains in one single chromatogram.

INTRODUCTION

Erythroid progenitor cells, such as BFU-E, have provided a good model for studying the developmental switching processes of human hemoglobin (Hb)

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[1, 2]. Among others, fetal Hb (Hb F) synthesis in BFU-E colonies has been studied in Saudi Arabian sickle cell anemia (SS) patients to get a better insight into the molecular mechanisms underlying the protective effect of high Hb F in these patients [3]. The biochemical analysis of Hbs synthesized by these cells have so far involved elaborate methods such as radioligand immunoassay [4] or a combination of size-exclusion, anion-exchange, and reversed-phase high-performance liquid chromatographic (HPLC) techniques [5]. Lysates of cells labeled with [^{3}H]leucine or [^{35}S]methionine mixed with non-radioactive Hb A and Hb F carriers have been analyzed by carboxymethylcellulose chromatography [6], DEAE-cellulose chromatography [7], or isoelectrofocusing (IEF) [2]. Furthermore, globin chain synthesis in these colonies has been evaluated by electrophoresis on polyacrylamide gels containing urea and Triton X-100 [1]. Huisman et al. [8] determined the $^{G}\gamma$ and $^{A}\gamma$ values (%) by analyzing the tryptic digests of Hb in lysates of radiolabeled BFU-E-derived cells.

Here we describe the use of HPLC methodology for analyzing the various Hb components that are synthesized in erythroid progenitor cells. Effective and reproducible separations were obtained that could be helpful in studying the biosynthesis of Hb in these progenitor cells. This method made it possible to observe variations in Hb F levels in SS patients with different β^{S} haplotypes [9] and encouraged us to analyze the relative quantities of the various globin chains synthesized in the progenitor cells obtained from ten SS patients and a similar number of their heterozygous (AS) relatives along with a few normal control subjects.

EXPERIMENTAL

Methylcellulose culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells, a very small fraction of which is comprised of BFU-E cells, were isolated from freshly collected blood samples (in heparin) on a Ficoll® gradient. They were plated in methylcellulose culture medium containing fetal bovine serum, deionized bovine serum albumin, β-mercaptoethanol, erythropoietin, and Mo-T cell line-conditioned medium as a source of burst-promoting activity as described by Miller et al. [10]. The plates were incubated in 5% humidified carbon dioxide at 37 °C for fourteen to fifteen days. Hemoglobinized BFU-E colonies were isolated by 'plucking' using a drawn-out Pasteur pipet and resuspending the cells in 0.5–1.00 ml Dulbecco's phosphate-buffered saline without calcium and magnesium. The cells were washed twice in cold saline followed each time by centrifugation at 13 600 *g* for 10 min at 4 °C in a Fisher Model 235B microcentrifuge. The cell pellet was lysed in about 50 μl of cold distilled water using sonication with an alternate freeze–thawing cycle. The cellular debris was removed by centrifugation at high speed for 10 min at 4 °C and the supernatant stored at 4 °C until used.

Separation of globin chains by reversed-phase HPLC

A slight modification of the method described by Shelton et al. [11] using a 250 mm × 4.6 mm large-pore Vydac C_4 column was applied [12]. The HPLC equipment comprised a Model 712 WISP sample processor, Lambda Max Model 481 LC spectrophotometer, and Model 510 HPLC pump controlled by an automated gradient controller (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.). The two mobile phases were mixtures of acetonitrile–water–trifluoroacetic acid (TFA). Mobile phase A: acetonitrile–water (60:40) with TFA added to a final concentration of 0.1%. Mobile phase B: acetonitrile–water (20:80) with 0.1% TFA. The column was equilibrated for 10 min with 50% mobile phase A (plus 50% mobile phase B), whereafter some 10–200 μl hemolysate containing approximately 50–100 μg of Hb was applied. The first step (T_1) in the development of the chromatogram was through the application of a gradient of mobile phase A (50→60%) with a corresponding decrease in mobile phase B (50→40%) resulting in an acetonitrile gradient (40→44%) for a period of 120 min. T_2 was a gradient of 60→80% mobile phase A (44→52% acetonitrile) applied for an additional 60 min. The column was finally purged with 100% mobile phase A (60% acetonitrile) for 5 min and then reequilibrated for 10 min with 50% mobile phase A. The flow-rate was maintained at 1 ml/min, the effluent was monitored at 220 nm, and the calculation of the relative concentrations of the polypeptides was made with a Waters 740 data module. Slight variations in the gradient system were sometimes made to facilitate the analyses.

RESULTS

Fig. 1 illustrates reversed-phase HPLC chromatograms of globin chains of peripheral blood hemolysates and BFU-E colony lysates from SS patients. Besides the heme component, β^S, pre-β^S, and α chains, minute quantities of the two γ chains ($^G\gamma$ and $^A\gamma$) can be observed in the red cell lysates. The $^G\gamma$ level in patient L.F. and the $^A\gamma$ level in patient M.N. are significantly higher than the corresponding levels in patient J.Fu. The relative amounts of the two γ chains in the BFU-E lysates appear to be higher than those for the corresponding red cell lysates except for patient M.N. where the levels are the same. Minute quantities of additional peaks can be seen in the BFU-E lysates. All these remain unidentified; however, one had the exact same chromatographic mobility as the embryonic ζ chain (data not shown but obtained by mixing these lysates with a similar lysate of heme-stimulated K562 cells that contain among others the embryonic Hb Gower-I or $\zeta_2\gamma_2$; see ref. 14).

Similar data but for a Hb S heterozygote are shown in Fig. 2. The two β chains (β^A and β^S) are incompletely separated, while γ chains are absent in the red cell lysate. The BFU-E lysate, however, was found to contain a significant

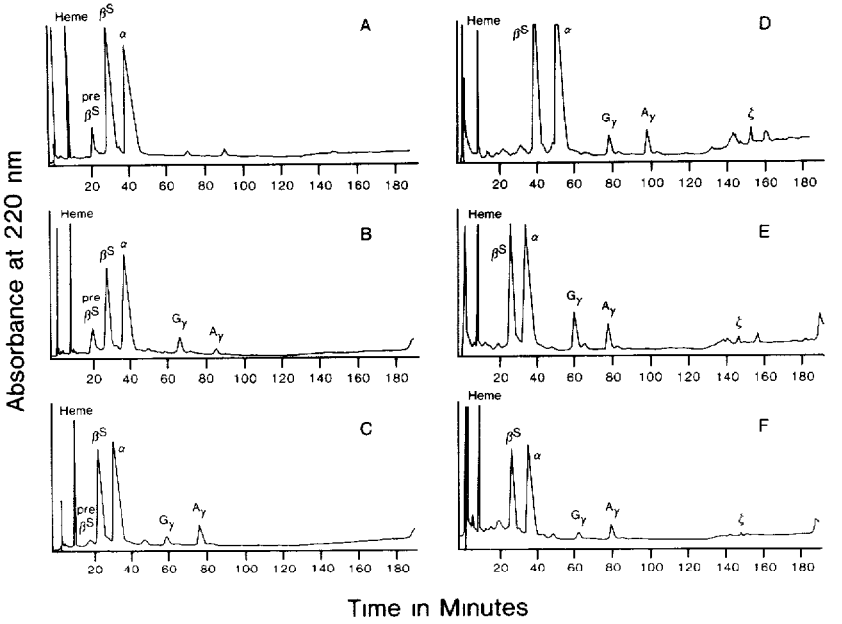


Fig. 1. Reversed-phase HPLC chromatograms of globin chains A, B and C illustrate the chromatograms of globins in whole blood hemolysates of SS patients J.Fu. (haplotype 19/19), L.F. (haplotype 3/3), and M.N. (haplotype 19/Mor), respectively, while D, E and F show similar analyses for the corresponding BFU-E lysates.

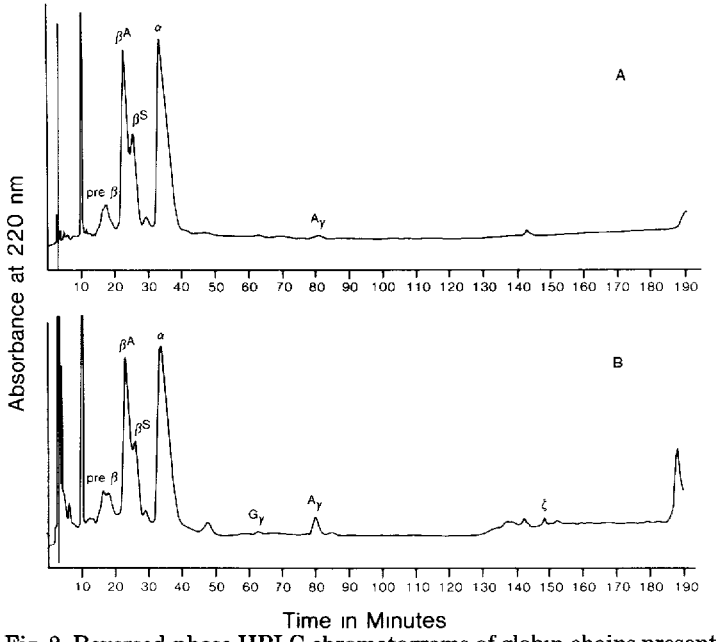


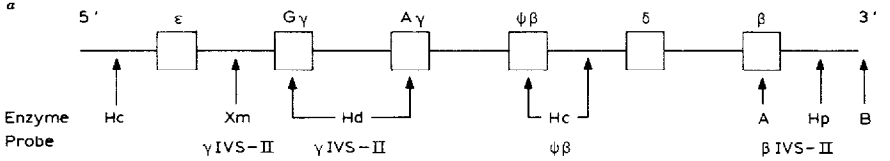
Fig. 2. Reversed-phase HPLC chromatograms of globin chains present in a red cell lysate (A) or a BFU-E lysate (B) from a subject with a Hb S heterozygosity.

TABLE I

GLOBIN CHAIN QUANTITATIVE DATA

Subject	Sex	Age (years)	β^S Haplotype[a]	Blood		BFU-E		
				γ^b (%)	$^G\gamma^c$ (%)	γ^c (%)	$^G\gamma^c$ (%)	ζ^c (%)
SS subjects								
L.Wa.	F	13	19/19	3.7	34.1	6.8	22.3	N.D.[d]
D.Wa	M	14	19/19	6.0	36.5	8.6	22.5	3.4
J.Fu.	F	6	19/19	5.4	46.7	13.6	32.5	2.6
S.H.	F	11	19/19	5.4	46.4	14.5	38.6	1.0
K.Wd.	F	28	3/19	15.8	59.3	26.5	56.4	N.D.
B.Wd.	M	38	3/19	6.2	59.9	35.6	49.2	N.D.
J.W.	F	5	3/19	19.6	57.2	14.5	32.1	2.4
T.W.	M	17	3/3	7.4	67.7	26.7	53.8	1.4
L.F.	F	46	3/3	15.0	72.7	27.6	59.1	1.6
M.N.	M	17	19/Mor	21.5	29.2	17.9	30.0	0.9
AS subjects								
C.Wa.	F	39	19	0.5	20.0	16.5	N.D.	N.D.
S.Fu	F	28	19	0.2	30.5	10.5	18.5	2.1
M.N.J.	M	48	19	0.6	43.7	2.9	N.D.	N.D
V.N.	F	23	19	0.4	41.8	2.6	N.D.	0.3
B.W.	F	40	3	0.5	77.8	11.2	64.6	2.1
V.W.	F	20	3	0.3	68.7	28.0	57.1	2.7
M.J.	F	40	Mor	4.6	10.5	13.8	27.3	1.2
N.J.N.	M	24	Mor	1.1	5.1	6.8	12.8	1.0
W.J.	F	57	Mor	0.9	5.3	9.0	20 6	1.0
L.J.	F	19	Mor	3.0	6.7	15.7	13.4	1.7
Normal controls								
P.G.	F	29		1.2	74.2	8.6	72.5	4.1
L C.	M	9		1.1	55.7	5.4	49.9	N.D.

[a]



Haplotype (β^S chromosome)

	Hinc II	Xmn	Hind III	Hind III	Hinc II	Hinc II	Ava II	Hpa I	Bam HI
19	–	–	–	–	–	+	+	–	+
20	–	–	+	–	–	–	+	+	+
3	–	+	+	–	+	+	+	+	+
31	+	+	+	–	+	+	+	+	–
Mor	+	–	–	–	–	–	+	–	+

[b]Determined by alkali denaturation [13] for the AS and control subjects.
[c]Determined by reversed-phase HPLC [11, 12]. $^G\gamma = 100 \cdot {}^G\gamma / [{}^G\gamma + {}^A\gamma]$; $\gamma\,(\%) = 100 \cdot \gamma / [\beta + {}^G\gamma + {}^A\gamma]$; $\zeta\,(\%) = 100 \cdot \zeta / [\zeta + \alpha]$.
[d]N.D. = not detectable

level of $^{A}\gamma$ with a minute amount of $^{G}\gamma$ and (perhaps) ζ. The other minor peaks remain unidentified.

Quantitative data for the $^{G}\gamma$ and ζ chains are listed in Table I. The list also includes the total γ chain level (i.e. $^{G}\gamma$ and $^{A}\gamma$). Often the low levels made quantitation less than optimal (particularly for the AS subjects; see Fig. 2). The patients are listed according to the haplotypes of the β^{S} chromosome(s), that are detailed in the bottom part of this table (the haplotype data are from ref. 9).

DISCUSSION

The chromatograms depicted in Figs. 1 and 2 clearly show the applicability of this reversed-phase HPLC procedure to the identification (and quantitation) of the globin chains in BFU-E lysates. The procedure requires extremely small quantities of Hb (the minimum quantity is some 50 μg). However, if the experiment permits the use of larger quantities (200–400 μg), this is to be preferred because of the low quantities of some of the chains (mainly $^{G}\gamma$, $^{A}\gamma$, ζ). Identification of the chains is, for obvious reasons, only possible by comparison of their elution times with those of known globin chains. The presumed identity of the ζ chains, that elute at the end of the chromatogram, was made that way. The method has several advantages over other chromatographic and electrophoretic methods such as speed and sensitivity; moreover, all globin chains can be detected and quantitated in one single chromatogram.

The data given in Table I provide some initial information about the globin chain synthesis in the in vitro BFU-E system for SS patients with different haplotypes and for their AS relatives. As shown before (but for a different haplotype) the BFU-Es from SS patients produce two to three times more Hb F than expected from their levels in peripheral cells [3]. This in vitro system of progenitor cell cultures apparently has the capability to increase both the $^{G}\gamma$ and $^{A}\gamma$ synthesis. This is even more evident from the data for the AS relatives; the Hb F levels in their BFU-E cultures were many times higher than the Hb F levels in the circulating red cells. The presence of minute quantities of ζ chains might change our thinking somewhat; perhaps erythroblasts are present in the BFU-E colonies representing an earlier stage in the erythroid differentiation pathway that produce not only increased quantities of embryonic chains but also γ chains. It would be of interest to search these BFU-E colonies for the presence of ϵ chains; unfortunately, this polypeptide chain elutes between the $^{G}\gamma$ and $^{A}\gamma$ chains and it will be difficult to detect this chain in chromatograms like the ones shown in Fig. 1.

The SS patient with the so-called Mor haplotype (subject M.N. of Table I) and his four AS relatives deserve some special attention. The BFU-E colonies from this SS patient consistently had equal or lower Hb F values than the percentage seen in his peripheral blood. It is of interest to mention that Gilman

et al. [15] recently discovered in these persons a C→T mutation at position −202 relative to the Cap site in the $^{A}\gamma$ gene. This mutation was considered to be directly responsible for the increased levels of $^{A}\gamma$ and total γ chains in the blood of this patient and in the blood samples of his AS relatives. His BFU-E colonies apparently do not have the capability of further increasing this $^{A}\gamma$ synthesis, in contrast to his AS relatives who showed the same Hb F levels in the BFU-E colonies as those of similar colonies of AS adults with other β^{S} haplotypes, while their low Hb F values were only moderately increased.

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