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A COMPARISON OF TWO PROCEDURES USEFUL FOR THE ISOLATION OF Hb F FROM ADULT RED BLOOD CELLS AND FOR THE QUANTITATION OF THE TYPES OF γ CHAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Two methods have been used to isolate Hb F from red cells with low levels of Hb F (<2% F_{AD}), namely an alkali denaturation procedure, as described by Tsuchiya *et al.* [*Dokkyo J. Med. Sci.*, 10 (1983) 13], and anion-exchange chromatography. Analyses of these Hb F enriched hemoglobin solutions by high-performance liquid chromatography allowed quantitation of the different γ chains in the Hb F. Although the data showed considerable variation, particularly for samples with low levels of Hb F, the final results obtained with the two approaches were comparable, suggesting that the much simpler and more economical alkali denaturation procedure can be used for this purpose.

INTRODUCTION

The determination of the relative quantities of the different types of γ chain [i.e., the $^{G\gamma}$ (75Ile, 136Gly), the $^{A}\gamma^{I}$ (75Ile, 136Ala), and the $^{A}\gamma^{T}$ (75Thr, 136Ala) chains] in the fetal hemoglobin (Hb F) of the normal adult is complicated by the low quantity of Hb F present in these blood samples [usually less than 1% (ref. 1)]. In the past, we determined the $^{G}\gamma$ to $^{A}\gamma$ (*i.e.*, the sum of the $^{A}\gamma^{I}$ and $^{A}\gamma^{T}$ chains) ratio using a chemical procedure which required at least 10–30 mg Hb F. Such a quantity of Hb F was obtained from one pint of blood through a series of chromatographic isolations[2]. Although this approach gave reliable and reproducible data it obviously is not applicable to routine analyses. The development of high-performance liquid chromatography (HPLC) for the separation of hemoglobin (Hb) chains[3–6] reduced the quantity of Hb F required for such analyses. In a large series of experiments Huisman and associates[7–9] isolated the Hb F from relatively small volumes (10–20 ml) of blood of normal adults, β-thalassemia heterozygotes, and subjects with a Hb S heterozygosity using preparative DEAE-cellulose chromatography followed by an additional purification of the Hb F containing Hb fraction with an analytical type

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of DEAE-cellulose chromatography[10]. In some instances the results were most acceptable; however, the chromatographic separation of the γ chains was often difficult to evaluate as extraneous protein zones overlapped the γ chain zones. This phenomenon occurred particularly when the Hb F level in the original blood sample was less than 0.5–0.8%. Attempts to further purify the Hb F with a specific HPLC procedure were only partially successful[9].

Recently Tsuchiya *et al.*[11] applied a well-known alkali denaturation procedure for the removal of the major portion of adult Hb, and next analyzed the Hb F by HPLC. These authors obtained adequate data using only 1–2 ml of blood. In this communication we describe the results of a comparative study involving adult red cell lysates as well as artificial mixtures with slightly elevated levels of Hb F. We conclude that the γ chain composition data obtained for Hb F being isolated either by alkali denaturation or by anion-exchange chromatography are about the same. However, the two approaches do not always provide a Hb solution with an adequate amount of Hb F to allow an accurate quantitation of the three types of γ chain.

MATERIALS AND METHODS

Blood samples

Approximately 10 ml of blood from each donor was collected by venipuncture in vacutainers using EDTA as anticoagulant. Twelve donors participated; nine were Hb S heterozygotes, two were heterozygous for β-thalassemia, and one had no apparent Hb abnormality. In addition, one cord blood sample, used in some mixing experiments, was selected because its Hb F contained three types of γ chains in a ratio of 13.8 ($^{A}\gamma^{T}$) to 69.2 ($^{G}\gamma$) to 16.0 ($^{A}\gamma^{I}$) as determined by HPLC (see below). Red cell lysates were prepared in the usual manner[12] and stored at 4°C for a period not exceeding 14 days.

Methods

All samples were analyzed by cellulose acetate and citrate agar electrophoresis[12]. The Hb F level was determined by the alkali denaturation procedure of Betke *et al.*[13].

Hb F enrichment by alkali denaturation. This method followed the directions given by Tsuchiya *et al.*[11]. One ml red cell lysate with 8–12 g Hb/dl was mixed with 20 ml of Drabkin's solution (for composition, see ref. 12), and the mixture kept at room temperature for 2 min. Next, 4 ml 1.2 *M* sodium hydroxide were added while mixing, followed by 4 ml saturated ammonium sulfate which were added exactly 2 min later. The precipitate was removed by centrifugation, the pH of the supernatant adjusted to about 7, and the dilute Hb solution filtered through a millipore filter (type HA 0.45 μm) whereafter the filter was washed three times with 0.9 g sodium chloride/dl. The dilute Hb solution, *i.e.*, the combined filtrates, was concentrated by filtration through a Diaflo ultrafiltration membrane under excess pressure with nitrogen (Amicon, 90 p.s.i. type 8200; with the YM10 62 mm membrane). The concentrated Hb solution (usually less than 0.5 ml with 0.8–1.5 g Hb/dl) was stored at 4°C until used. The notation F_{AD} will be employed when reference is made to this solution. If it is desirable to isolate a larger quantity of the F_{AD} solution the procedure can be modified to include a larger volume of red cell lysate and increased volumes of reagents.

Hb F enrichment by DEAE-cellulose chromatography. This method has been described in detail before[10]. Some 4–8 ml red cell lysate with 8–12 g Hb/dl were chromatographed on a 20 × 2 cm preparative column using a sodium chloride gradient system in a 0.2 *M* glycine–0.01% potassium cyanide developer starting with 0.005 *M* sodium chloride and with 0.02 *M* sodium chloride in the second developer. The fraction containing the minor Hb A_1 and Hb F components was isolated, and rechromatographed on an 1.5 × 0.9 cm column using the same sodium chloride gradient system. The fraction containing Hb F (as well as some Hb A and Hb A_1) was eluted from the DEAE-cellulose with an 0.2 *M* sodium chloride (in 0.2 *M* glycine–0.01% sodium chloride) developer whereafter the solution was concentrated by ultrafiltration as described above for the F_{AD} solution. The F_{chrom} solution (usually less than 0.5 ml with 1–2 g Hb/dl) was stored at 4°C until used.

Quantitation of the three types of γ chain. The HPLC procedure used in these experiments has been described in detail[4].

Preparation of artificial mixtures. Mixtures were prepared from a normal red cell lysate (with 0.43% Hb F, determined by alkali denaturation) and the cord blood red cell lysate listed above. The final Hb F levels in the five mixtures were determined by alkali denaturation[13].

RESULTS

The chromatographic separation of the three types of γ chain in Hb solutions with low Hb F levels

As much as 600 μg Hb, dissolved in 0.2–0.3 ml, was applied to the microcolumn. Fig. 1 illustrates two chromatograms; the chromatogram in the top section concerned about 350 μg Hb of a Hb F_{AD} solution which contained approximately 4% Hb F (the Hb F_{AD} was isolated from the artificial mixture of normal Hb A and a minute quantity of cord blood red cell lysate). The large α and β chain peaks were only partially separated, and the three (minor) γ chain peaks appeared as broad zones in the latter part of the chromatogram. A similar result was obtained when some 600 μg Hb of a Hb F_{chrom} solution was analyzed (bottom section of Fig. 1; the F_{chrom} solution contained about 3% Hb F). The broad α and β zones overlapped each other as well as the heme peak. The three γ chain zones were separated from each other while the $^{G}\gamma$ peak was partially resolved into two distinct zones (this was observed in numerous chromatograms). Quantitation of the three γ chains was reproducible although the standard deviations (S.D.) were rather large (Table I).

A comparison of the γ chain percentages in F_{AD} and F_{chrom} solutions isolated from red cell lysates with low levels of Hb F

The acceptable reproducibility justified the application of the techniques to red cell lysates of adults with low levels of Hb F. Table II provides the $^{G}\gamma$ data for nine individuals. A reasonable agreement existed in six cases; however, considerable differences were observed in the samples from donors 2, 4 and 7, preventing a classification of the $^{G}\gamma$ level as being either of the adult or newborn type[14–16]. The $^{G}\gamma$ levels in F_{AD} preparations of three additional Hb S heterozygotes agreed with similar data obtained some 10 years ago using a more extensive chemical procedure[2].

These rather unsatisfactory data prompted two additional series of experi-

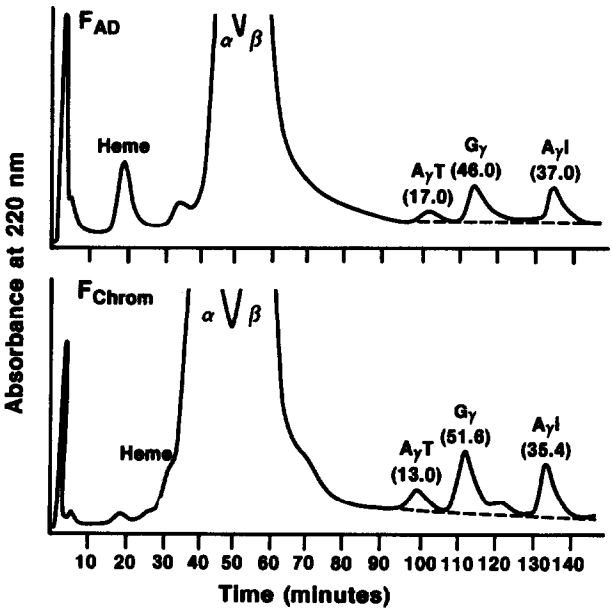

Fig. 1. The separation of the three types of γ chain by HPLC. F_{AD} refers to a Hb F enriched solution prepared by alkali denaturation and F_{chrom} to one prepared by DEAE-cellulose chromatography. The numbers between parentheses are percentages. The original samples used in the isolation procedures contained 0.85% Hb F by alkali denaturation. The two Hb F enriched Hb solutions contained between 3 and 4% Hb F, calculated from the computer printout. See text for further details.

ments, namely an evaluation of the effect of the level of Hb F (between 0.8 and 5%) as well as storage time (up to two weeks at 4°C) on the percentages of the three types of γ chain in F_{AD} and F_{chrom} solutions.

The relative quantities of the three γ chains in artificial mixtures with increasing levels of Hb F.

These mixtures (M-1 through M-6) were prepared by combining appropriate volumes of a normal red cell lysate and of a cord blood red cell lysate containing Hb F with an $^{A}\gamma^{T}$:$^{G}\gamma$:$^{A}\gamma^{I}$ ratio of 13.8:69.2:16.0. The normal red cell lysate contained a low level of Hb F (0.43%); its γ chain composition was not determined. The per-

TABLE I

AVERAGES AND STANDARD DEVIATIONS FOR THE QUANTITIES OF THE THREE γ CHAINS IN ARTIFICIAL MIXTURES DETERMINED BY HPLC

*Mixture**	*% Hb F (alk. den.)*	*n*	*Enrichment procedure*	$^{A}\gamma^{T}$ *(%)*	$^{G}\gamma$ *(%)*	$^{A}\gamma^{I}$ *(%)*
M-2	1.34	5	F_{AD}	14.1 ± 2.1	48.7 ± 3.1	37.2 ± 5.1
M-2	1.34	5	F_{chrom}	13.8 ± 2.6	50.9 ± 3.0	35.3 ± 4.6
M-5	4.03	5	F_{AD}	17.6 ± 1.8	56.8 ± 3.3	25.6 ± 3.4
M-5	4.03	5	F_{chrom}	17.4 ± 3.5	57.4 ± 3.2	25.2 ± 3.4

* The preparation of the mixture is described in the text.

TABLE II

THE PERCENTAGES OF $^{G}\gamma$ CHAIN IN Hb F-ENRICHED Hb SOLUTIONS

F_{AD}, enriched by alkali denaturation; F_{chrom}, enriched by DE-52 chromatography. n.d. = not determined.

Blood donor	*Condition*	*% Hb F (alk. den.)*	*% $^{G}\gamma$ (F_{AD} sol.)*	*% $^{G}\gamma$ (F_{chrom} sol.)*	*$^{G}\gamma$* level*
1	AS	0.60	27.5	31.9	Adult
2	AS	0.54	52.6; 45.3**	69.8	?
3	AS	0.36	46.5	32.6	Adult
4	AS	0.56	52.3	68.1	Newborn
5	AS	0.62	21.8	31.9	Adult
6	AS	n.d.	32.6; 46.6**	38.0	Adult
7	AA	0.43	61.4; 66.9**	40.6	?
8	A-βTh.	6.04	29.2	43.8	Adult
9	A-βTh.	2.60	58.4	64.6	Newborn

* This $^{G}\gamma$ level is labelled "adult" when the $\%^{G}\gamma$ chain varies between 25 and 45%, and "newborn" when it varies between 55 and 70% (as in the Hb F of newborn babies).

** Duplicate analyses on the same Hb F enriched sample.

centages of Hb F in the six mixtures (alkali denaturation) were 0.84 (M-1), 1.34 (M-2), 1.85 (M-3), 2.52 (M-4), 4.03 (M-5), and 4.75 (M-6). Hb F was isolated from each mixture using the two methods outlined above, and the relative quantities in the various F_{AD} and F_{chrom} solutions were determined by HPLC. The results of these analyses are summarized in Fig. 2. The following observations can be made. (a)

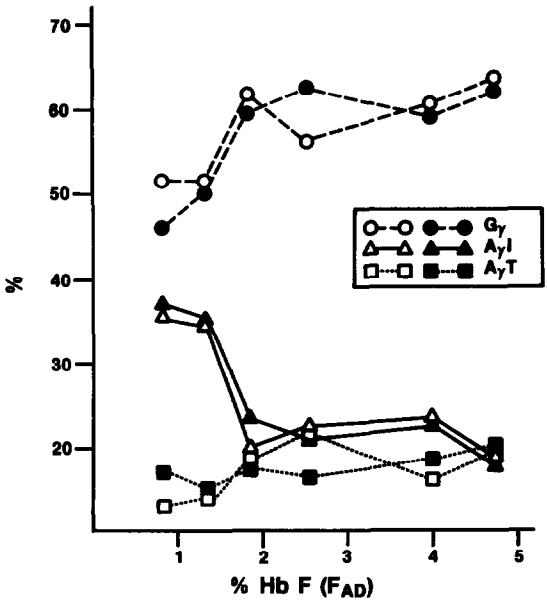

Fig. 2. The levels of the three types of γ chain in the Hb F isolated from mixtures of red cell lysates with different levels of Hb F. ●—●, Hb F_{AD} or Hb F solutions enriched by alkali denaturation. O—O, Hb F_{chrom} or Hb F solutions enriched by DE-52 chromatography.

TABLE III

THE EFFECT OF STORAGE AT 4°C ON THE LEVELS OF THE THREE TYPES OF γ CHAIN IN Hb F ENRICHED Hb SOLUTIONS

F_{AD}, enriched by alkali denaturation; F_{chrom}, enriched by DE-52 chromatography.

Type of Hb solution	*% Hb F (alk. den.)*	*Enrichment procedure*	*Days of storage*	$^{A}\gamma^{T}$ (%)	$^{G}\gamma$ (%)	$^{A}\gamma^{I}$ (%)
M-2*	1.34	F_{AD}	0	13.8	47.2	39.0
			2	14.7	54.4	30.9
			8	15.0	51.1	33.9
			9	11.1	65.6	23.3
M-2	1.34	F_{chrom}	0	15.1	46.4	38.5
			4	9.5	60.2	30.3
			15	10.1	45.4	44.5
M-5*	4.03	F_{AD}	0	14.8	56.7	28.5
			7	13.9	58.8	27.3
			8	12.5	59.6	27.9
M-5	4.03	F_{chrom}	1	14.3	59.0	26.7
			15	13.5	59.5	27.0

* Mixtures prepared from a normal red cell lysate and a cord blood red cell lysate containing the three types of γ chain (see text).

Although the percentages in some mixtures (M-1, M-4, M-5) showed considerable variation it was evident that the data for the F_{AD} and F_{chrom} solutions were comparable. (b) The M-1 and M-2 mixtures, *i.e.*, the mixtures with minute additions of the cord blood red cell lysate, had relatively high levels of $^{A}\gamma^{I}$ chains (35%), and low levels of $^{A}\gamma^{T}$ (15%) and $^{G}\gamma$ (50%) chains. In contrast, F_{AD} and F_{chrom} solutions isolated from the M-4, M-5 and M-6 mixtures, with a considerably higher quantity of cord blood Hb F, had nearly constant levels of $^{A}\gamma^{T}$ (18%), $^{G}\gamma$ (60%), and $^{A}\gamma^{I}$ (22%) chains. The difference between these data is due to the contribution by the Hb F present in the normal red cell lysate which probably contained the $^{A}\gamma$ and $^{G}\gamma$ chains in a 6:4 ratio, as seen in most adult individuals[2,6].

The effect of time of storage at 4°C

This was evaluated for two F_{AD} and two F_{chrom} solutions, both being isolated from two of the artificial mixtures. Table III provides further details of the experiments. The values for all three types of γ chain varied considerably for the F_{AD} as well as the F_{chrom} solutions, when prepared from the mixture with the low Hb F level (M-2). This was not the case for similar Hb F solutions isolated from the mixture with the higher Hb F level (M-5). The latter results suggest that both solutions can be stored for 1 to 2 weeks without a change in the relative quantities of the three types of γ chain.

CONCLUSIONS

The results presented here will lead to the following conclusions:

(a) The HPLC data obtained for the Hb solutions enriched in Hb F through alkali denaturation or through anion-exchange chromatography are comparable.

Thus, the alkali denaturation enrichment procedure is to be preferred as it is faster and more cost-effective.

(b) The data obtained by this procedure for blood samples containing 1.5–2% Hb F or more are reproducible and appear reliable although repeated analyses gave average data with rather large standard deviations (Tables I). Despite these limitations, the method can be used for blood samples of patients with conditions characterized by (slightly) elevated levels of Hb F, such as (most types of) β-thalassemia, $\delta\beta$-thalassemia, and various forms of HPFH.

(c) The method can also be applied to blood samples with low levels of Hb F (less than 1–1.5%) but a greater variability can be expected. Acceptable data probably requires repeated analyses of the same samples as slight variations in the small γ chain zones in the HPL chromatogram will have a considerable effect on the final calculations.

(d) It seems desirable to develop additional micro methods which will allow an improved separation of Hb F from Hb A and its Hb A_1 related minor components.

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