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# Quantities of adult, fetal and embryonic globin chains in the blood of eighteen- to twenty-week-old human fetuses

FERDANE KUTLAR, HUGO MOSCOSO, CHARLES R. KIEFER and FREDERICK A. GARVER

*Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-2100 (U.S.A.)*

SINAN BEKSAÇ and LÜTFI ÖNDEROGLU

*Department of Obstetrics and Gynecology, Hacettepe University, Ankara (Turkey)*

AYTEMIZ GURGEY and ÇIGDEM ALTAY

*Children's Medical Center, Hacettepe University, Ankara (Turkey)*

and

TITUS H. J. HUISMAN*

*Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA 30912-2100 (U.S.A.)*

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## ABSTRACT

The prenatal diagnostic program, established at Hacettepe University in Ankara for the purpose of detecting $\beta$-thalassemia ($\beta$-thal), sickle cell anemia (SS), and Hb S-$\beta$-thal, offered the opportunity of evaluating the relative quantities of adult ($\beta^A$, $\beta^S$), fetal ($^G\gamma$, $^A\gamma$, $^A\gamma^T$), and embryonic ($\varepsilon$, $\zeta$) chains in 26 fetuses, aged 18–20 weeks. Methodology involved micro high-performance liquid chromatographic (HPLC) procedures and immunology using an mAb, specific for the embryonic $\varepsilon$ chain. A good correlation was observed between the $\beta/\gamma$ *in vitro* chain synthesis ratio and the level of $\beta^A$ and/or $\beta^S$ chains determined by reversed-phase HPLC; the combination of these two sets of data strengthens the prenatal diagnostic approach of detecting $\beta$-thal major but not $\beta$-thal trait. The levels of the different $\gamma$ chains were about as observed in newborn babies; the frequency of the $^A\gamma^T$ variant in the 26 fetuses was the same as observed for a larger group of Turkisch newborn babies. The level of the embryonic $\zeta$ chain was higher than seen in full-term babies and varied between 0 and 1.3%; 5 of the 26 fetuses showed the complete absence of $\zeta$. The embryonic $\varepsilon$ chain was not detectable, not even in babies with $\beta$-thal major. These data indicate that the synthesis of $\varepsilon$ is completely turned off in fetuses at the age of 18–20 weeks, while that of $\zeta$ continues, albeit at a low level.

## INTRODUCTION

Analyses providing information about the relative quantities of the different globin chains in the blood of human fetuses are usually limited to those comparing the *in vitro* synthesis of $\alpha$ chains, the $\beta$ chains of hemoglobin A (Hb A), and the $\gamma$ chains of Hb F (reviewed in ref. 1). The development of accurate and rapid microchromatographic procedures using small columns and a rela-

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tively high pressure high-performance liquid chromatography (HPLC) has allowed the separation and quantitation of numerous types of globin chains [2–4]. Moreover, the formation of monoclonal antibodies against the embryonic $\zeta$ [5–7] and $\varepsilon$ (ref. 8 and this paper) chains has made it possible to quantitate these chains with great accuracy. Thus, a combination of these two analytical tools will allow an evaluation of all globin chains present at any stage of development. The establishment of prenatal diagnostic programs which use fetal blood for the diagnosis of certain forms of thalassemia and other hemoglobinopathies has made it possible to collect small volumes of fetal blood useful for such studies. This paper provides data for 26 fetuses from whom blood was obtained during the second trimester of pregnancy. The possible presence of the $\varepsilon$ chain was studied to see whether the synthesis of this chain is completely turned off in the second trimester or if there is any delay at this age, particularly in the babies with $\beta$-thalassemia ($\beta$-thal) major. This question is pertinent since the level of the embryonic $\zeta$ chain was significantly higher in babies with an $\alpha$ chain deficiency.

## EXPERIMENTAL

### *Blood samples*

These were "leftovers" of prenatally collected samples as part of a large program to identify $\beta$-thal major or sickle cell anemia (SS) in fetuses, aged 18–20 weeks. Sampling was by fetoscopy and the *in vitro* chain synthesis was by CM-cellulose of $^3$H-labeled globin chains (for a review of methodology, see refs. 1 and 9). The 26 samples studied here had specific $\beta/\gamma$ synthetic ratios on which the $\beta$-thal diagnosis was based, namely 0–0.2 for $\beta$-thal major, 0.021–0.050 for $\beta$-thal trait or perhaps normal, and 0.051 and higher for normal. The diagnosis of a $\beta^S$ homozygosity (SS) was based on the relative chromatographic mobilities of the $\beta^S$ and $\beta^A$ chains in the CM-cellulose chromatographic system. All 26 samples were stored as washed red cells at $-20$°C and transported by one of the authors by air from Ankara, Turkey to Augusta, GA, U.S.A. Twenty-three of the 26 samples were pure fetal samples, while some 20% maternal blood contamination was present in three (samples 12, 24, and 26). Adult blood contamination was evaluated by establishing a red cell distribution curve with the channelyzer of the Coulter counter (two distinct sizes are seen when adult red cells are also present) and by the Hb F staining procedure of Kleihauer and Betke (fetal cells are fully colored while adult cells are empty; the method is described in ref 10).

### *Methods*

Hematological data were obtained immediately after collection of each sample using an automatic cell counter. Red cell lysates were prepared by freezing and thawing followed by high-speed centrifugation in a refrigerated centrifuge. Some 0.2–0.5 ml of hemolysate was available for each sample.

Reversed-phase HPLC (RP-HPLC) with the large-pore $C_4$ column was used

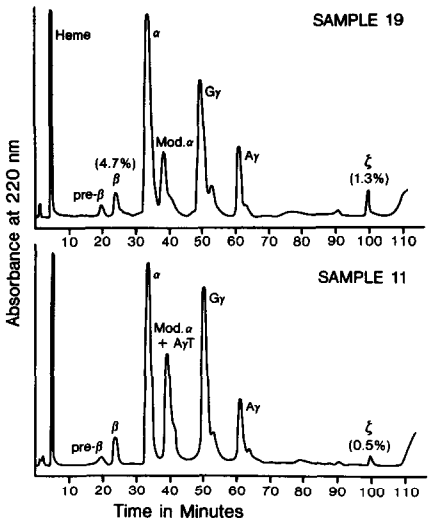


Fig. 1. Separation of the globin chains by RP-HPLC. The pre-$\beta$ and $\beta$ zones are combined for the calculation of total $\beta$. The same is done for the modified $\alpha$. The elution times of some modified $\alpha$ and $\gamma$ chains are similar to that of the $^{A}\gamma^{T}$ variant so that these zones are recovered together (bottom chromatogram). The two eluents were: A [60% acetonitrile–40% water–0.1% trifluoroacetic acid (TFA)] and B (20% acetonitrile–80% water–0.1% TFA). Gradient: 48% A→58% A in 70 min, followed by 58% A→78% A in 35 min.

to separate and quantitate the globin chains. A slow gradient allowing a complete separation of the $\alpha$, $\beta$, and $\gamma$ chains and of the embryonic $\zeta$ chain was used for all samples [4]. Fig. 1 provides two examples. The $\beta$ chain is eluted first, followed by the $\alpha$ chain and the $\gamma$ chains; the normally occurring $^{G}\gamma$ and $^{A}\gamma$ chains were present in all samples, while the $^{A}\gamma^{T}$ mutant which elutes between the $\alpha$ and $^{G}\gamma$ chains was observed in ten samples. The $\zeta$ chain was eluted last at an elution time of $\sim 100$ min. Quantitation of this embryonic chain (as percentage of total $\alpha + \zeta$) was accurate at the 0.1% level [4].

Despite optimal storage conditions some changes had occurred resulting in a faster moving $\beta$ chain component (pre-$\beta$) and slower eluting $\alpha$, $^{G}\gamma$, and $^{A}\gamma$ components. Calculation of the relative quantities of these chains included the modified components (the type of modification was not established but was considered to be glycation of all chains, acetylation of the $\gamma$ chains, and perhaps partial oxidation of methionyl groups in the chains). No significant change in the mobility of the $\zeta$ chain was observed; previous data [11] had also indicated that $\zeta_2\gamma_2$ is a stable tetramer.

It was unfortunate that some of the modified chains and the $^{A}\gamma^{T}$ variant did not separate in this system (Fig. 1, second chromatogram). This made it necessary to isolate Hb F by micro DEAE-cellulose chromatography [10] whereafter the

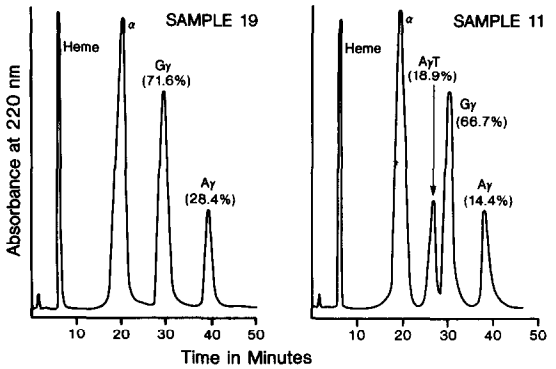


Fig. 2. Quantitation of the three types of γ chain, *i.e.* $^{A}\gamma^{T}$, $^{G}\gamma$, and $^{A}\gamma$, by RP-HPLC in isolated Hb F. The same eluents were used; the gradient was 48% A→58% A in 50 min.

relative quantities of the two or three types of γ chains could readily be determined (Fig. 2).

*Quantitation of the embryonic ε chain*

The possible presence of (small) quantities of this chain could not be determined by RP-HPLC because the ε chain elutes between the $^{G}\gamma$ and $^{A}\gamma$ chains in this sytem. Therefore, its possible presence was evaluated with an immunological procedure.

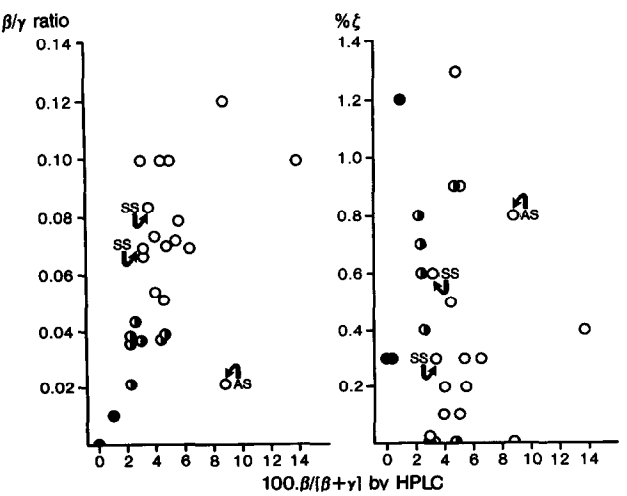


Fig. 3. Possible relationships between the relative quantities of the β chain and the *in vitro* β/γ synthesis ratio (left) and the relative quantities of the embryonic ζ chain (right).

*Production of antibodies*

The hybridoma production system used was that of Taggart and Samloff [12]. The immunogen was Hb Gower-I ($\alpha_2\varepsilon_2$), isolated from culture fluids of the cell line K562 (kindly provided by A. E. Felice). Two subcutaneous doses, three weeks apart, each of 100 $\mu$g Hb Gower-I emulsified in complete, then incomplete Freund's adjuvants, were administered to a 15-week-old RBF/Dn female mouse. After a 19-day rest, two intravenous doses were given, two days apart, each of 10 $\mu$g Hb Gower-I, with sacrifice and fusion on the following day. The fusion procedure used was that of Lane *et al.* [13]. Screening of culture fluids for $\varepsilon$-specific antibodies employed the horseradish peroxidase (HRP)–2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) enzyme-linked immunosorbent assay (ELISA) system previously described [14] to determine selective reactivity for Hb Gower-I *versus* Hb Portland-I ($\zeta_2\gamma_2$). Hb Portland-I was isolated by DEAE-cellulose chromatography [10] from a red cell lysate of a fetus with hydrops fetalis. The clone of interest, $\varepsilon$-1-276, was typed as IgG1, $\kappa$ using the MonoAb-ID EIA Kit A (Zymed Labs., South San Francisco, CA, U.S.A.). Concentrated monoclonal antibody, as ascites fluid, was produced in a 4-month-old BALB/c mouse which had been primed with pristane (2,6,10,14-tetramethylpentadecane), 0.5 ml intraperitoneally (i.p.), then immunosuppressed 16 days later with cyclophosphamide, 250 mg/kg i.p., followed one day later by i.p. injection of $10^7$ hybridoma cells.

*Characterization of the mAb*

The specificity of the mAb was determined by reacting various dilutions of the ascites fluid with 1 $\mu$g of Hbs Gower-I, Portland-I, F, A, and $A_2$. The indicator system utilized antimouse HRP and the substrate tetramethylbenzidine. Absorbances were read at 630 nm.

*ELISA for quantification of the $\varepsilon$ chain*

Hb Gower-I was diluted in a normal cord blood hemolysate to obtain percentages from 10 to 0.005%. Aliquots of 100 $\mu$l of these standards (1 mg/ml Hb) were coated to the wells of microtiter plates (Costar, Cambridge, MA, U.S.A.) and reacted with a 1:5000 dilution of the mAb and with the indicator system. The sensitivity of the ELISA was 0.05% or 0.05 $\mu$g per well.

## RESULTS

*Hematological data*

Table I lists the $\beta/\gamma$ ratios for three fetuses with a $\beta$-thal major, for seven fetuses with a possible $\beta$-thal trait, for thirteen fetuses without a $\beta$-thal, for two fetuses with SS, and one with an Hb S trait. None of the $\beta$-thal mutations were identified; the first two fetuses listed in Table I were twins with a $\beta$-thal homozygosity. Hematological data could be determined for seven fetuses, three with a $\beta$-thal trait, three normal fetuses, and one with SS. Hb levels varied between 10.6

TABLE I

HEMATOLOGICAL DATA AND THE RELATIVE QUANTITIES OF GLOBIN CHAINS AS DETERMINED BY RP-HPLC

| Condition[a] | *n* | $\beta/\gamma$ ratio | Hb (g/dl) | PCV (l/l) | RBC ($10^{12}$/l) | MCV (fl) | MCH (pg) | MCHC (g/dl) | $\frac{\beta}{\beta+\gamma}$ | Data by HPLC (%)[b] | | | $\frac{\zeta}{\alpha+\zeta}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | $^{G}\gamma^{c}$ | $^{A}\gamma^{c}$ | $^{A}\gamma^{Tc}$ | |
| $\beta$-Thal major | 1 | 0.000 | | | | | | | 0.0 | 70.0 | 30.0 | 0 | 0.3 |
| | 2 | 0.000 | | | | | | | 0.0 | 70.7 | 29.3 | 0 | 0.3 |
| | 3 | 0.010 | | | | | | | 1.0 | 71.8 | 28.2 | 0 | 1.2 |
| $\beta$-Thal trait | 4 | 0.038 | 11.3 | 0.35 | 4.1 | 130 | 42 | 32 | 2.4 | 71.1 | 28.9 | 0 | 0.7 |
| | 5 | 0.034 | 11.8 | 0.36 | 2.8 | 130 | 42 | 32 | 2.4 | 69.2 | 11.8 | 19.0 | 0.6 |
| | 6 | 0.025 | | | | | | | 2.3 | 69.4 | 13.4 | 17.2 | 0.8 |
| | 7 | 0.037 | | | | | | | 3.0 | 70.4 | 20.6 | 9.2 | 0 |
| | 8 | 0.039 | | | | | | | 4.7 | 73.0 | 27.0 | 0 | 0 |
| | 9 | 0.039 | 10.6 | 0.31 | 3.8 | 120 | 41 | 34 | 4.6 | 70.4 | 29.6 | 0 | 0.9 |
| | 10 | 0.044 | | | | | | | 2.6 | 67.7 | 12.7 | 19.6 | 0.4 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Normal | 11 | 0.100 | | | | | | | 4.3 | 66.7 | 14.4 | 18.9 | 0.5 |
| | 12 | 0.099 | | | | | | | 13.8 | 70.6 | 29.4 | 0 | 0.4 |
| | 13 | 0.100 | | | | | | | 5.0 | 69.0 | 31.0 | 0 | 0.1 |
| | 14 | 0.120 | | | | | | | 8.8 | 69.9 | 10.5 | 19.6 | 0 |
| | 15 | 0.069 | 11.3 | 0.34 | 2.7 | 126 | 42 | 33 | 6.3 | 69.1 | 30.9 | 0 | 0.3 |
| | 16 | 0.067 | | | | | | | 3.1 | – | – | – | 0 |
| | 17 | 0.072 | 11.6 | 0.36 | 4.3 | 121 | 39 | 32 | 5.4 | 70.3 | 13.4 | 16.3 | 0.3 |
| | 18 | 0.074 | | | | | | | 3.9 | 75.2 | 8.1 | 16.7 | 0.1 |
| | 19 | 0.051 | 10.9 | 0.33 | 2.4 | 139 | 45 | 32 | 4.7 | 71.6 | 28.4 | 0 | 1.3 |
| | 20 | 0.070 | | | | | | | 4.8 | 67.7 | 0 | 32.3 | 0.9 |
| | 21 | 0.100 | | | | | | | 2.9 | 70.3 | 29.7 | 0 | 0 |
| | 22 | 0.054 | | | | | | | 4.0 | 67.2 | 11.7 | 21.1 | 0.2 |
| | 23 | 0.079 | | | | | | | 5.7 | 71.3 | 28.7 | 0 | 0.2 |
| SS | 24 | 0.084 | 11.7 | 0.34 | 2.8 | 121 | 42 | 35 | 3.5 | 76.2 | 23.8 | 0 | 0.3 |
| | 25 | 0.069 | | | | | | | 3.1 | 69.2 | 30.8 | 0 | 0.6 |
| AS | 26 | 0.041 | | | | | | | 8.8[d] | 70.4 | 29.6 | 0 | 0.8 |

[a] Diagnosis based on $\beta/\gamma$ *in vitro* chain synthesis ratio (0–0.02: $\beta$-Thal major; 0.02–0.05: $\beta$-Thal trait; 0.051 and higher: normal) and on the presence of the $\beta^S$ chain.
[b] Examples of chromatograms are given in Fig. 1.
[c] Determined for Hb F isolated by DEAE-cellulose chromatography [10].
[d] $\beta^A + \beta^S$.

and 11.8 g/dl; the red cells were large (MCV 121-139 fl) and fully hemoglobinized (MCHC 32-35 g/dl). No difference was seen between the three normal fetuses and the three with a $\beta$-thal trait.

*Chromatographic data*

Table I also lists this information for the same 26 samples. The data include the relative quantities of $\beta$ chain (as percentage of total $\beta + \gamma$), the quantities of the different $\gamma$ chains ($^{G}\gamma$, $^{A}\gamma$, $^{A}\gamma^{T}$), and that of the $\zeta$ chain. The $\beta$ levels varied from 0 to 13.8%; most values fell between 2 and 6%. The $^{G}\gamma$ levels varied between 66.7 and 76.2% which are comparable to those observed for newborn babies. Ten fetuses carried the $^{A}\gamma^{T}$ variant; one fetus (No. 20) was homozygous for this mutation. The $\zeta$ chain was detectable in 21 of the 26 samples; its quantity varied from 0.1 to 1.3%. No attempt was made to define the possible presence of an $\alpha$-thal in any of these samples.

*Immunological data*

The characterization of the mAb showed it to be specific for $\varepsilon$ chain without interference or cross-reactivity with $\alpha$, $\beta$, $\gamma$, $\delta$, or $\zeta$ chains. All 26 samples were analyzed for the possible presence of the embryonic $\varepsilon$ chain with a sensitivity of 0.05% of the total hemoglobin (Hb) present. None of the samples were positive. Additional data for 83 samples from subjects, some with various hematological abnormalities including two fetuses with hydrops fetalis, eleven normal newborns, twenty-four normal adults, thirteen adults with $\beta$-thal trait, ten adults with $\alpha$-thal-2 trait, six adults with Hb H disease, and the remainder with various disorders, were also negative. K562 cells lysates served as positive controls in these experiments.

## DISCUSSION

The data presented here concern hematological values and globin chain composition data for blood samples collected from human fetuses aged 18–20 weeks. The blood samples were collected during a prenatal diagnostic program at Hacettepe University, intended to detect homozygosity for $\beta$-thal, SS, or Hb S-$\beta$-thal. The methodology was CM-cellulose chromatography which allows an evaluation of the relative synthesis of (abnormal) $\beta$ chains, $\gamma$ chains, and $\alpha$ chains, and which has been most successful for its intended purpose. The present study has extended the data obtained and provides information about the relative quantities of all globin chains, including the embryonic $\varepsilon$ and $\zeta$ chains.

The limited hematological information, listed in Table I, did not allow any differentiation between a normal condition and a $\beta$-thal heterozygosity at this stage of development. Some of the quantitative globin chain data, given in Table I, are replotted in Fig. 3. An acceptable correlation appears to exist between the $\beta/\gamma$ *in vitro* chain synthesis ratios and the relative quantities of $\beta$ chain (as percent-

age of $\beta + \gamma$). Most fetuses had $\beta$ chain levels between 2 and 6%, The three fetuses diagnosed as having $\beta$-thal major had the expected low levels of 0–1%, while five of the seven fetuses with a possible $\beta$-thal heterozygosity had $\beta$ chain levels between 2 and 3%. Samples from three fetuses contained $\beta$ chain levels of 8–13.8%; contamination with maternal blood was demonstrated for two (samples 12 and 26).

The levels of $^{G}\gamma$ in the Hb F averaged 70.3% (range 66.7–76.2%) which is the same as the levels observed for normal full-term babies and babies with SS [15,16]. The $^{A}\gamma^{T}$ variant, *i.e.* the $^{A}\gamma$ chain with an Ile→Thr substitution at position 75 [17], was present in ten babies (nine heterozygotes and one homozygote); this corresponds to a frequency of 0.212 which is similar to that found for a large group of full-term Turkish babies [18,19]. There was a considerable variation in the relative amounts of the $^{A}\gamma$ chain (average 13.0%) and the $^{A}\gamma^{T}$ chain (average 17.5%) for the nine heterozygotes; this variation is difficult to explain and might in part be due to the condition of the samples.

Of the embryonic globin chains ($\varepsilon$ and $\zeta$) only the $\zeta$ chain could be identified and quantitated in 21 of the 26 samples. None of the samples contained any $\varepsilon$ chain suggesting that at the fetal age of 18–20 weeks the synthesis of this embryonic chain is completely turned off. The average value for $\zeta$ of 0.45% (range 0–1.3%) is distinctly higher than that found in normal full-term Turkish babies (average value of $0.17 \pm 0.23$ for 157 babies) and more comparable to the values seen in full-term babies with varying $\alpha$ chain deficiencies such as the $\alpha$-thal-2 homozygosity ($-\alpha/-\alpha$) or the $\alpha$-thal-1 heterozygosity ($--/\alpha\alpha$) [4]. It would be of interest to continue similar analyses for blood samples collected at other stages of human development.

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