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DETECTION OF THE EMBRYONIC ζ CHAIN IN BLOOD FROM NEWBORN BABIES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography (RP-HPLC) using the large-pore Vydac C_4 column has been used to detect and quantitate the embryonic ζ chain in blood samples of normal babies and of newborns with varying degrees of α chain deficiencies. The ζ chain eluted at the end of the chromatogram at about 130 min using a modified and extended gradient. Its identity was confirmed by structural analysis of ζ chain isolated from a blood sample of a fetus without active α globin genes, *i.e.* with hydrops fetalis (– –/– –). The quantity of ζ in normal babies is less than 0.7% [% of (α + ζ)] and is dependent upon the maturity of the baby as it was only present in babies with low levels of β chain or hemoglobin (Hb) A. The presence of a ζ globin gene deletion [A. E. Felice *et al., Hum. Genet.*, 73 (1986) 221; and P. Winichagoon *et al., Nucleic Acids Res.*,10 (1982) 5853] did not affect the level of ζ in the newborn. All babies with an α-thalassemia-2 heterozygosity, *i.e.* with three active α globin genes or –α/αα, had ζ in a range of 0.1–0.9%; again the level showed a negative correlation with that of the β chain. Newborns with an α-thalassemia-2 homozygosity or –α/–α had a varying level of ζ of 0.3–2.3%, which did not correlate with the level of β, suggesting that ζ chain production persists after birth in this condition. Macrochromatographic analyses in combination with RP-HPLC indicated that the ζ chain is present as $\zeta_2\gamma_2$ or Hb Portland-I, as expected.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has become an important tool in the study of the minor globin chains that may be present in lysates of red blood cells from human newborns and adults. Recently, we identified the $^{M}\gamma$ chain as a newly discovered γ globin chain which resembled the $^{A}\gamma$ chain of the fetal hemoglobin (Hb F) but with a Leu→Met substitution at position γ^{141} (refs. 1 and 2). This $^{M}\gamma$ chain was found, among others, in blood samples from numerous subjects with different forms of hereditary persistence of Hb F (HPFH) and in patients with sickle cell anemia[1,2]. During that study, we observed two minor components (peaks) in extended HPLC chromatograms of newborn red cell lysates. One

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of these was suspected of being the embryonic ζ chain as it occupied a position at the end of the chromatogram, comparable to that reported by others using somewhat different HPLC systems[3–5]. This ζ chain is closely related to the α chain in primary structure (the ζ chain, like the α chain, is 141 amino acids long but differs at 57 positions[6]). It is mainly present during the first three months of fetal life, after which it is replaced by α. Its synthesis is regulated by one ζ globin gene which is located about 3.5 kilobases (kb) 5′ to a nonfunctional pseudo (ψ) ζ globin gene, and about 8 kb 5′ to the two α globin genes on the short arm of chromosome No. 16 (refs. 7 and 8). Hb Portland-I or $\zeta_2\gamma_2$ (ref. 9) has been found in considerable quantities in blood of newborn babies without active α globin genes, *i.e.* hydrops fetalis, and to a lesser extent in newborns with less severe α chain deficiencies.

This communication describes the identification of the minor zone, its occurrence in red cell lysates from newborn babies with and without different types of α chain deficiency, and its quantitative relationship with the level of the β chain in these cell lysates, assuming this to be some measure of fetal maturity[10].

MATERIALS AND METHODS

Blood samples

Cord blood samples were collected in vacutainers with EDTA as anticoagulant, and transported within one week to the laboratory in Augusta, GA, U.S.A. The samples were from normal babies without a detectable hemoglobinopathy except for the presence of variable quantities of Hb Bart's (γ_4). Most babies were born in local hospitals or in hospitals within the State of Georgia. A few samples were received from institutions in cities on the west coast of the United States, and from Nanning, People's Republic of China. Most babies were Black, some were Caucasian, and a few were Chinese or Vietnamese. Consent to collect the samples was obtained.

Hb analyses

All samples were analyzed by cellulose acetate electrophoresis at alkaline pH; samples with an α or β or γ chain variant were not included in the study. The presence of Hb Bart's (γ_4) was indicated for each sample as negative or undetectable, as small (less than *ca.* 5%), as large (5–10%), and high (over 10%). Quantitation of Hb Bart's was by microcolumn chromatography, as described before[11].

RP-HPLC

The method of Shelton *et al.*[12] was used for the separation of the globin chains. About 50 μg Hb was applied onto a 250 × 4.1 mm I.D. Vydac C_4 column, and the chromatogram was developed with a gradient between solution A [acetonitrile–water (60:40) with 0.1% trifluoroacetic acid (TFA) in final concentration] and solution B [acetonitrile–water (20:80) with 0.1% TFA]. The first gradient was 50% A to 60% A in 80 min, and the second was 60% A to 78% A in 60 min at a flow-rate of 1 ml/min.

Preparative RP-HPLC made use of a Bio-Sil TSK-ODS-120T (C_{18}) column (300 × 21.5 mm I.D.). The amount of Hb applied varied from 6 to 8 mg. The developers were developer A or acetonitrile–water (52:48) (0.1% TFA), developer B or acetonitrile–water (35:65) (0.1% TFA) and developer C or acetonitrile–water

(60:40) (0.1% TFA). The gradients applied were 57% A (+ 43% B) to 69% A (+ 31% B) in 150 min, followed by 69% A (+ 31% B) to 85% A (+ 15% B) in 70 min, and 74% C (+ 26% B) to 78% C (+ 22% B) in the last 80 min. The flow-rate was maintained at 6 ml/min.

Isolated ζ chain was digested with trypsin (TPCK Trypsin, Worthington, U.K.) overnight at pH 8.9 and at 37°C. The resulting soluble peptides were separated by RP-HPLC[13] and their amino acid compositions determined with an automated Beckman Spinco Model 121M amino acid analyzer. Data obtained were compared with compositions of peptides as provided in refs. 6 and 14.

In some experiments, the Hbs in red cell lysates were chromatographed on 30 × 2 cm I.D. DEAE-cellulose columns as described before[15]. The various Hb components (Hb A, Hb F, and the minor Hb components which eluted behind Hb F) were concentrated by filtration under pressure, and next analyzed by starch gel electrophoresis[16], and by RP-HPLC on the Vydac C_4 column (see above).

DNA analyses

DNA was isolated from white blood cells using the method of Poncz *et al.*[17]. The number of α and ζ globin genes was determined as described before[18,19]. In these gene mapping procedures, use was made of the following restriction enzymes: Eco RI, Bam HI, Xba I, Bgl II, and Hind III, and of the following two probes: 1.5 kb Pst I fragment pRBα1 and a 0.5 kb Pst I fragment pHζcDNA. A normal condition with four α globin genes will be indicated as αα/αα; any α-thal-2 trait as −α/αα; any α-thal-2 homozygosity as −α/−α (including double heterozygosity for the two different types of α-thal-2, −4.2 kb and −3.7 kb); any α-thal-1 heterozygosity as − −/αα; an Hb H disease as − −/−α; and hydrops fetalis or α-thal-1 homozygosity as − −/− −. Abnormalities in ζ globin genes were rare; when observed, these will be mentioned for each individual case.

RESULTS

Chromatographic detection of the ζ chain

Fig. 1 compares five chromatograms of red cell lysates from newborn babies without and with an increasingly severe α globin gene deficiency, as indicated. All five chromatograms have a (minor) peak, identified as ζ, which elutes between 125–130 min. The ζ zone is preceded by an unknown component, marked X in Fig. 1. The quantity of ζ varies considerably; when expressed as % of (α + ζ) it is present from 0.6 to 100% in the baby with hydrops fetalis (additional quantitative data are given later). The presence of the ζ chain is particularly marked in the babies with only two (or less) active α globin genes. Baby No. 9279, with an α-thal-2 homozygosity (−α/−α), had 1.74% ζ [as % of (α + ζ)] and 9.1% Hb Bart's or γ_4 (as % of total Hb); baby No. 9453 with a Hb H disease (− −/−α) had 4.36% ζ and 15% Hb Bart's; and the hydrops fetalis baby HF, without any active α globin genes (− −/− −), had 100% ζ and nearly 80% Hb Bart's. Thus, the level of the ζ chain, like that of the α chain lacking Hb Bart's[20], is a measure of the severity of the α chain deficiency. The presence of a notable quantity of ζ in red cells of normal baby No. 10903 (αα/αα) and baby No. 11655 with an α-thal-2 trait (−α/αα) is of interest; data to be presented below will show that the ζ chain is detectable in about 50% of all normal newborns, and in all babies with the α-thal-2 heterozygosity.

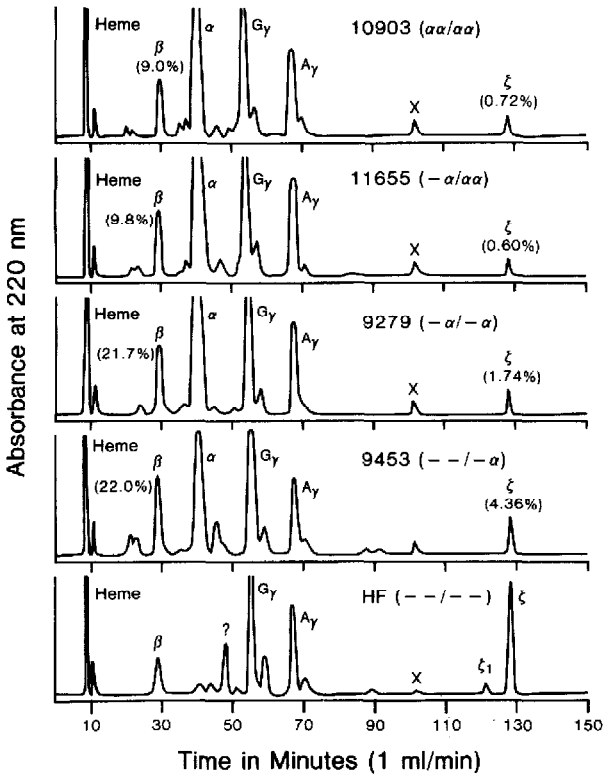

Fig. 1. Separation of globin chains by RP-HPLC using a large-pore Vydac C_4 column. All five samples are from newborn babies with the stated α globin gene deficiencies (HF = baby with hydrops fetalis). Several known chains are identified as α, ζ, β, or $^{G}\gamma$ and $^{A}\gamma$ chains. The %β is calculated as % of (β + γ); the %ζ is also calculated as % of (α + ζ).

Identification of the ζ chain

A larger quantity of the ζ chain was isolated from the red cell lysate of the hydrops fetalis baby HF (− −/− −) using the larger Bio-Sil TSK-ODS-120T (C_{18}) column. Fig. 2 illustrates the separation that was obtained; material from the ζ peak (protein zone No. 10) of four such chromatograms was combined for use in the structural analyses. Peptides from a tryptic digest were separated and isolated by RP-HPLC (data not shown), and their amino acid compositions were determined. These data, summarized in Table I, offer convincing evidence to conclude that the protein present in the ζ peak is indeed the embryonic ζ globin chain. It is, therefore, assumed that the components observed in identical position in HPLC chromatograms of other red cell lysates are the same as this embryonic chain.

Isolation of the ζ chain as Hb Portland-I

Both CM-cellulose and DEAE-cellulose macrochromatography were used for this purpose; data from DEAE-cellulose chromatograms were most informative. One such chromatogram is shown in Fig. 3. The sample used was cord blood red cell lysate from a baby with an α-thal-2 homozygosity (−α/−α), which contained 9.1% Hb

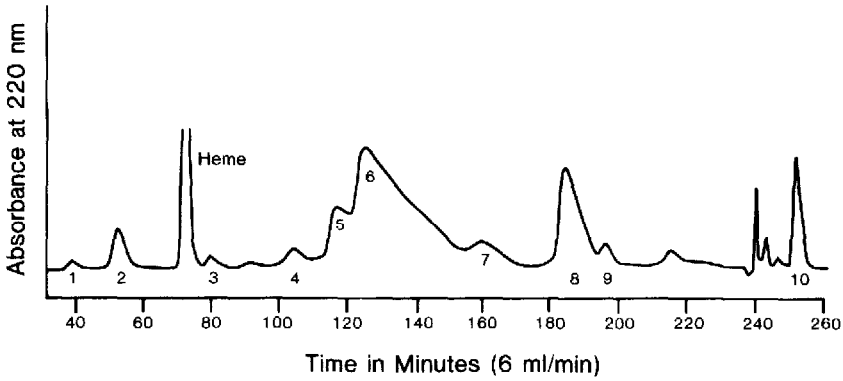

Fig. 2. Separation of globin chains on a preparative RP-HPLC column (C_{18}). Approximately 9 mg Hb from baby HF was applied. Several protein zones (Nos. 1, 2, 3, 4, and 5) remain unidentified. The chromatogram should be compared with that obtained on an analytical C_4 column given in Fig. 1. Material from protein zone No. 10 was used for structural analysis.

Bart's or γ_4. Besides the expected Hb zones A and F_0, three minor zones (labelled 1, 2 and 3) were isolated, which were assumed to contain Hb F_1 or fetal Hb with acetylated γ chains[15] and the α chain lacking Hb Bart's or γ_4 (ref. 20). Starch gel electrophoresis (insert of Fig. 3) identified component No. 1 (present for 9.7%) as being mainly Hb A like (Hb F_1 has a mobility similar to Hb A in this system), and component No. 3 (present for 6.6%) as being primarily the fast-moving Hb Bart's.

TABLE I

AMINO ACID COMPOSITION OF SOLUBLE TRYPTIC PEPTIDES OF THE ζ CHAIN*

Amino acid	*T-2*	*T-4*	*T-5*	*T-6*	*T-7*	*T-8***	*T-9*	*T-15*
Aspartic acid		1.00(1)		1.14(1)		1.63(1)	2.22(2)	
Threonine	0.83(1)	3.22(4)	1.01(1)	0.97(1)				
Serine		0.95(1)	0.86(1)	1.28(1)	0.88(1)		1.95(2)	
Glutamic acid	1.03(1)	2.60(3)	1.12(1)	1.00(1)				
Proline			0.99(1)	2.03(2)				
Glycine		1.00(1)		1.14(1)	1.09(1)	1.24(1)	1.78(2)	
Alanine		1.00(1)		0.91(1)	0.93(1)	3.08(3)	1.03(1)	
Valine						3.03(4)		
Isoleucine		1.71(2)					1.93(2)	
Leucine		0.94(1)	2.06(2)	2.10(2)			1.03(1)	
Tyrosine				0.98(1)				0.98(1)
Phenylalanine			0.95(1)	1.71(2)				
Histidine			0.89(1)	2.27(2)	0.81(1)			
Lysine			1.00(1)		1.29(1)	1.00(1)	1.07(1)	
Arginine	1.17(1)	1.00(1)		0.90(1)				1.02(1)
Residues in ζ Chain	5–7	17–31	32–40	41–56	57–61	62–71	72–82	140–141

* In moles/peptides. Peptides 1, 3 and 13 were not isolated in pure form while peptides T-10, T-11, T-12, and T-14 were not observed in the HPLC chromatogram. Values between parentheses are expected numbers of residues from refs. 6 and 14.

** Rather impure; valine value is low due to Val–Val bond.

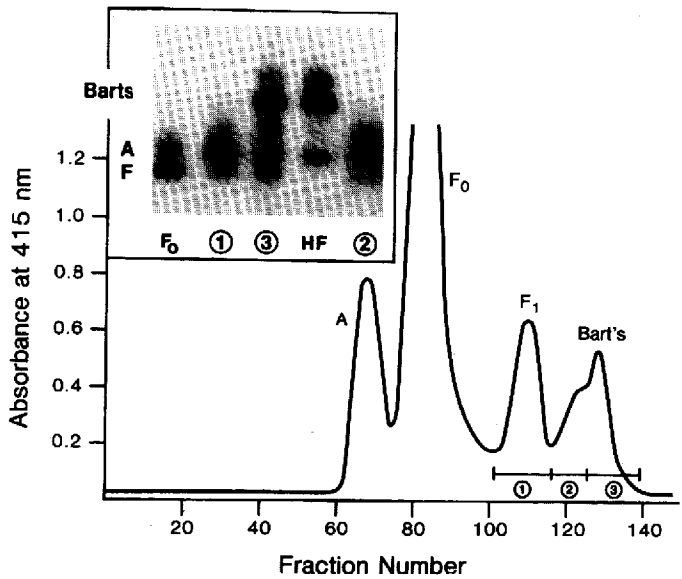

Fig. 3. Separation of Hb components by DEAE-cellulose macrochromatography. About 60 mg Hb was applied on a 30 × 2 cm I.D. column. The sample was from baby No. 9279 with an α-thal-2 homozygosity (–α/–α) and 9.1% Hb Bart's. Zones A, F_0, No. 1, No. 2, and No. 3 were isolated, concentrated, and analyzed by starch gel electrophoresis (insert), and by RP-HPLC (Fig. 4).

Component No. 2 (present for 4.4%) eluted as a shoulder, slightly ahead of component No. 3; its major Hb component had a mobility slightly faster than Hb A (insert of Fig. 3).

Considerably more information was obtained from the RP-HPLC chromatograms of the isolated zones (Fig. 4). Hb A (first chromatogram) was highly impure and contained both Hb A ($\alpha + \beta$) and Hb F ($\alpha + {}^{G}\gamma + {}^{A}\gamma$). The isolated Hb F_0 zone, however, was nearly pure; it contained nearly no β chain (second chromatogram). The ζ chain was absent. The Hb of zone No. 1 (third chromatogram) was a complex mixture; besides α chain it contained primarily modified β chain [β_1 is presumably glycosylated β (ref. 15)] and modified γ chains (${}^{G}\gamma_1$ and ${}^{A}\gamma_1$ are presumably the acetylated ${}^{G}\gamma$ and ${}^{A}\gamma$ chains). Normal β, ${}^{G}\gamma$, and ${}^{A}\gamma$ were also present, as was a small quantity of ζ [1.3% as % of ($\zeta + \alpha$)]. Zone No. 2 (fourth chromatogram) contained nearly no modified β and γ chains but α and ζ [ζ was about 1/3 of ($\alpha + \zeta$)] and ${}^{G}\gamma$ and ${}^{A}\gamma$, suggesting the presence of Hb F ($\alpha_2\gamma_2$), of Hb Portland-I ($\zeta_2\gamma_2$), and perhaps of a hybrid Hb ($\alpha\zeta\gamma_2$). Zone No. 3 (fifth chromatogram) contained primarily ${}^{G}\gamma$ and ${}^{A}\gamma$ (presumably in the form of Hb Bart's) with only small amounts of α and ζ chains. Based on the relative quantities of the Hb zones Nos. 1, 2 and 3, and the ζ chain percentages in these zones, it was possible to calculate the quantities of the ζ chain in each zone and in the total red cell lysate; the latter value [as mg ζ/100 mg ($\alpha + \zeta$)] was 1.65 mg, which is quite similar to the 1.74% observed in the whole red cell lysate of this newborn (third chromatogram of Fig. 1). It is worth noting that component X (Fig. 1) did not appear in the chromatograms of the isolated Hb fractions, although some material eluted at 90–95 min in some of the chromatograms (Fig. 4).

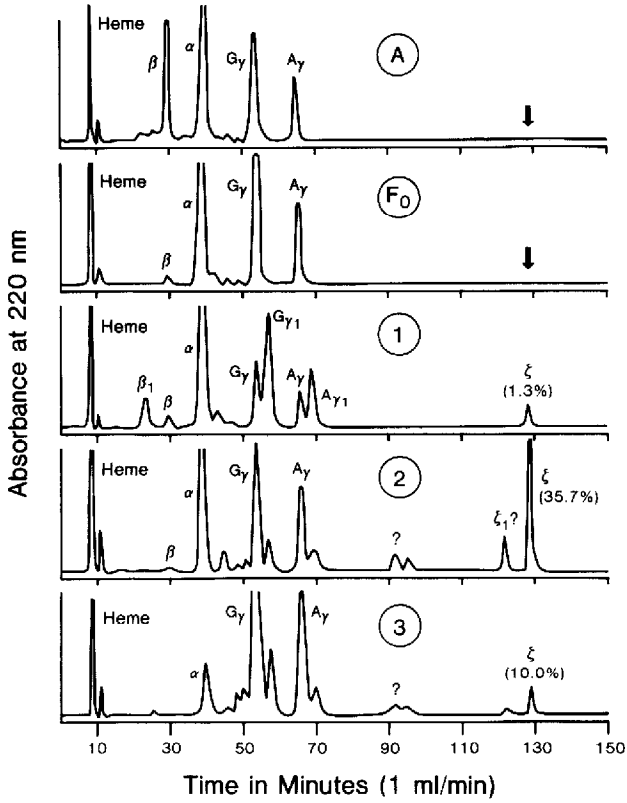

Fig. 4. Separation of globin chains by RP-HPLC using a large-pore Vydac C_4 column. The five samples are isolated Hb fractions from the DEAE-cellulose chromatogram shown in Fig. 3. Known chains are appropriately identified. The %ζ chain is calculated as % of (α + ζ).

TABLE II

RECOVERY OF THE ζ CHAIN FROM MINOR HEMOGLOBIN FRACTIONS ISOLATED BY DEAE-CELLULOSE CHROMATOGRAPHY

Case	*Condition**	*Bart's***	*%ζ Chain****			
			Fraction			*Hemolysate*
			1	*2*	*3*	
10395	−α/αα	1.7	0.4	4.9	2.5	0.61
11265	−α/αα	2.5	0	0.9	0	0
11244	−α/−α	5.6	0.2	11.2	3.5	0.46
8476	−α/−α	5.6	1.0	12.6	10.2	0.76
8550	−α/−α	8.9	0.3	7.6	2.2	0.98
9279	−α/−α	9.1	1.3	35.7	10.0	1.74
9453	− −/−α	15.0	4.4	25.4	12.5	4.36

* See text.

** By microcolumn chromatography.

*** $\%\zeta = 100 \cdot \zeta/[\alpha+\zeta]$.

Similar experiments were conducted for six additional cord blood samples (two from babies with $-\alpha/\alpha\alpha$; three from babies with $-\alpha/-\alpha$; one from a newborn with Hb H disease or $--/-\alpha$). The data, summarized in Table II, are similar to those obtained for baby No. 9279. Fraction No. 2 contained the ζ chain, but incomplete separation on the DEAE-cellulose column often resulted in a considerable overlap with the other minor Hb zones.

Quantitative aspects

Quantitative data were collected for 31 newborns with four active α globin genes ($\alpha\alpha/\alpha\alpha$), 19 with an α-thal-2 trait ($-\alpha/\alpha\alpha$), and 19 with an α-thal-2 homozygosity ($-\alpha/-\alpha$). All these babies were Black and lived in various communities in Georgia (U.S.A.). Eight of the 31 normal newborns had an additional ζ gene deletion. This genetic anomaly concerns a deletion of an 11 kb DNA fragment between the ζ and $\psi\zeta$ genes; the resulting chromosome carries one ζ globin gene with its 5′ end derived from the ζ gene and its 3′ end from the $\psi\zeta$ gene[19,21]. All babies with the α-thal-2 heterozygosity or homozygosity had the normal ζ globin gene arrangement. DNA data to support these results will not be presented here but will be published at a later time.

Fig. 5 compares the $\%\zeta$ with the level of Hb Bart's for all 69 newborns. Eleven of the 23 normal babies ($\alpha\alpha/\alpha\alpha$; no ζ deletion) had no detectable ζ chain, in four this level was 0.1% (*i.e.* the lower limit of detection), while an average value of 0.35% (range 0.2–0.7%) was observed for the remaining eight babies. All eight normal babies with a ζ globin gene deletion had a detectable ζ chain peak; its quantity averaged 0.3% (range 0.1–0.5%). These values were slightly higher for the 19 babies with the

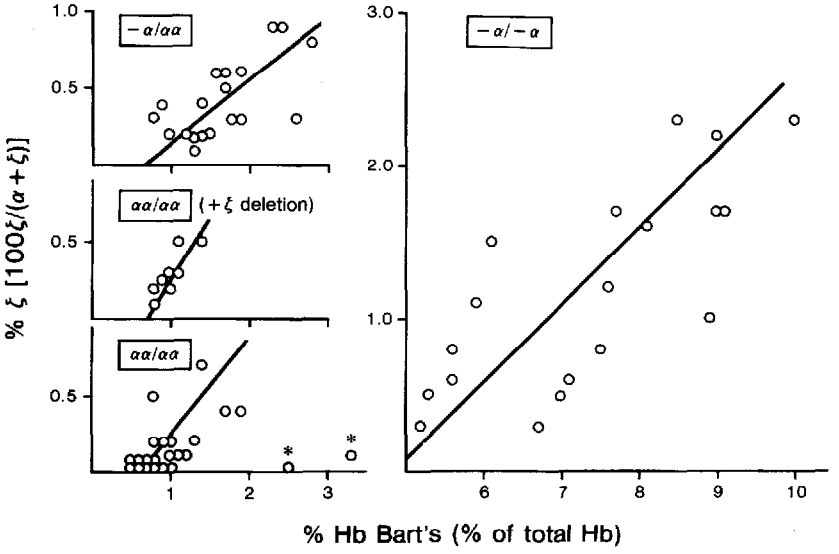

Fig. 5. The relationship between the levels of Hb Bart's (γ_4) and the ζ chain in blood from normal newborn babies, normal newborn babies with a ζ globin gene deletion, and newborns with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$) or homozygosity ($-\alpha/-\alpha$). The samples marked with an asterisk (*) were stored for two months prior to the analyses; the "Hb Bart's" level is probably too high, due to contaminants formed during storage.

α-thal-2 heterozygosity (–α/αα); the average value was 0.42% with a range of 0.1–0.9%. Considerably larger percentages were observed for the 19 babies with the α-thal-2 homozygosity (–α/–α) as their ζ level averaged 1.20% with a range of 0.3–2.3%. A direct correlation was present between the %ζ and the % Hb Bart's for all four groups of babies. This is not surprising because the Hb Bart's fraction, isolated by microchromatography, is a mixture of several proteins including Hb Bart's or γ_4, Hb Portland-I or $\zeta_2\gamma_2$, and some non-Hb proteins (the latter are responsible for about 0.5–0.8% of the "Hb Bart's" level).

Fig. 6 illustrates a possible relationship between the percentages of ζ chain [as % of (α + ζ)] and β chain [as % of (β + γ)] in the cord blood samples. The % β chain was readily calculated from the data provided by the RP-HPLC chromatogram (Fig. 1) and is considered to be a measure of fetal maturity. The data show higher ζ percentages at lower levels of β chain. Normal babies (αα/αα) with 15–20% β chain or higher, appear not to have ζ chain, while this value is slightly higher (25–30%) for babies with an α-thal-2 heterozygosity (–α/αα). Such a relationship was not evident for the babies with an α-thal-2 homozygosity (–α/–α).

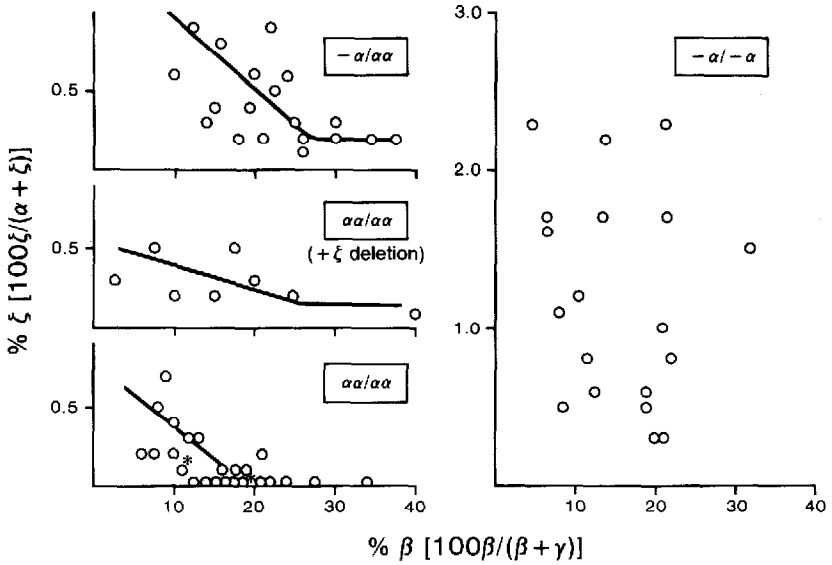

Fig. 6. The relationship between the levels of β chain and ζ chain in blood from normal newborn babies, normal newborn babies with a ζ globin gene deletion, and newborns with an α-thal-2 heterozygosity (–α/αα) or homozygosity (–α/–α). For the two samples with an asterisk (*) see legend of Fig. 5.

DISCUSSION

This study has offered structural and chromatographic evidence for the presence of variable quantities of the embryonic ζ chain in cord blood samples from all newborn babies with a mild to severe α chain deficiency and in many normal newborns. Its position in the RP-HPLC chromatogram is unique as it elutes very late, confirming earlier observations made with a different form of HPLC[3–5]. This ζ chain

is probably present in newborn red cells as Hb Portland-I or $\zeta_2\gamma_2$, which has specific chromatographic and electrophoretic properties (Fig. 3). The presence of this Hb in some minor Hb fractions, isolated from cord blood lysates by macrochromatography, has been difficult to evaluate because of its low quantity; identification of the globin chain composition by RP-HPLC, however, readily located the minute Hb Portland-I component. Theoretically, one can also expect the presence of Hb Portland-II or $\zeta_2\beta_2$ (ref. 4); its quantity in many cord blood red cell lysates, however, will be too low to allow its detection. A second peak (labelled X in Fig. 1) which elutes about 25 min ahead of the ζ chain was not detected in the Hb fractions isolated by DEAE-cellulose chromatography (Fig. 4). It may well be that it represents one of the many non-Hb proteins present in red cell lysates; it appears to be present in cord blood samples as well as in various lysates of adult red blood cells (data not shown).

Variable amounts of Hb Bart's (γ_4) are found in red cells of babies with the different forms of α-thalassemia, and microcolumn chromatography has been applied for its quantitation[11]. The fast-moving Hb zone, isolated by this method, which is comparable to the peaks 2 and 3 isolated by macrocolumn DEAE-cellulose chromatography (Fig. 3), contains besides Hb Bart's notable quantities of Hb Portland-I ($\zeta_2\gamma_2$), as evidenced from the levels of ζ chain in these fractions (Fig. 4). For instance, an increase in the quantity of the Hb Bart's fraction above 5% in red cell lysates of babies with an α-thal-2 homozygosity ($-\alpha/-\alpha$), appears to be primarily due to an increase in the production of the ζ chain (Fig. 5) which combines with γ to form Hb Portland-I. The slight increases in the level of "Hb Bart's", *i.e.* above the baseline value of 0.5–0.8%, in normal babies ($\alpha\alpha/\alpha\alpha$) and in babies with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$), are also caused by the presence of the ζ chain containing Hb Portland-I (Fig. 5).

Recently, Chui *et al.*[22] used a highly sensitive and specific radioimmunoassay (RIA) to detect ζ globin chains in blood from normal and α-thalassemic newborns. They observed an average value of 0.15% ζ in normal ($\alpha\alpha/\alpha\alpha$) babies (20% had no detectable ζ chain) and higher values in babies with notable quantities of Hb Bart's or γ_4. These investigators also found that the ζ chain disappears soon after birth in normal babies. These results are in close agreement with ours. We observed the ζ chain in 16 of the 31 normal babies tested; the lowest level of detection was 0.1% [expressed as % of ($\alpha + \zeta$)]. Among these 16 newborns were eight with a ζ globin gene deletion on one chromosome. As the RP-HPLC analysis also offers a satisfactory quantitation of the β chain, a comparison between the relative quantities of β and ζ chains made it possible to evaluate the existence of a negative correlation between these two parameters. Such a correlation was observed for normal babies ($\alpha\alpha/\alpha\alpha$) and babies with an α-thal-2 heterozygosity ($-\alpha/\alpha\alpha$) (Fig. 6). In both instances, the ζ chain level decreases with increasing β chain level, suggesting a direct relationship with fetal maturity. It is interesting to note that the disappearance of the ζ chain takes somewhat longer in the α-thal-2 baby than in the normal newborn; the ζ chain is not detectable in the normal baby when the β chain level has reached a level of 15–20%, and in the α-thal-2 heterozygous baby when this level approaches 25–30%. No such relationship was detected for the α-thal-2 homozygous ($-\alpha/-\alpha$) newborn baby (Fig. 6), suggesting a continuous synthesis of ζ, perhaps even into adult life.

Our limited data for babies with a ζ globin gene deletion, suggest functional ζ genes on both chromosomes because the ζ chain level in these babies at birth, was the same as that for normal babies without such a deletion (Fig. 6).

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