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Human embryonic ζ-globin chains in adult patients with α-thalassemias

(hemoglobin ontogeny/hemoglobin Portland/hydrops fetalis/β-thalassemias)

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ABSTRACT **Human embryonic ζ-globin chains are α-globin-like chains that are normally present during the first three months of gestation. In this investigation, ζ-globin chains measured by a specific and sensitive radioimmunoassay and by an electrophoretic technique were found to be present in all 7 patients studied with hereditary Hb H disease, and in 8 out of 24 patients with α-thalassemia trait. ζ-Globin chains were not detected in 20 other patients with β-thalassemia trait. These results suggest that the deletion of two α-globin genes on the same chromosome is accompanied by the continued expression of embryonic ζ-globin genes in adult individuals.**

α-Thalassemia is a hereditary disorder in which α-globin chain synthesis is either decreased or absent. It is usually caused by complete deletion of one or more α-globin genes, although partial deletion as well as nondeletion abnormalities have been described (1). In homozygous α-thalassemia with complete deletion of all four α-globin genes, the ζ-globin chains, normally present during the first 3 months of gestation, continue to be synthesized even in the third trimester (2–4). These ζ-globin chains combine with γ-globin chains to form Hb Portland-1 ($\zeta_2\gamma_2$), which accounts for about 10–15% of the hemoglobins present in infants with the Hb Bart's hydrops fetalis syndrome (5, 6). These infants are usually stillborn or die shortly after birth.

Hereditary Hb H disease is caused by deletion of either three α-globin genes or, rarely, two α-globin genes in combination with an abnormal α-globin gene such as α Constant Spring (1). α-Thalassemia trait is commonly the result of either the deletion of two α-globin genes from one chromosome or the deletion of one α-globin gene from each of the two homologous chromosomes (1). In this study, evidence is presented that the embryonic ζ-globin genes continue to be expressed in 7 patients with hereditary Hb H disease and in 8 out of 24 other patients with α-thalassemia trait.

MATERIALS AND METHODS

Hematological Data. Hematological parameters were determined by standard laboratory procedures, using a Coulter S electronic counter. Hemoglobin studies, including starch gel electrophoresis, determinations of Hb A_2 by microcolumn chromatography (normal range 1.8–3.3%) and Hb F by the Betke method (normal range 0.4–1.2%), were done as previously described (7). Hb H inclusion bodies in peripheral blood erythrocytes were carefully searched for under a light microscope, after the blood was incubated with brilliant cresyl blue (British Drug House) for 2 hr at 37°C (8).

DEAE-cellulose column chromatography (9) was performed with a number of patients' hemolysate samples and the α/β-globin synthetic ratio in peripheral blood reticulocytes was assessed in some patients (10). Hemolysates were electrophoresed in Triton X-100/urea/polyacrylamide gels (11), and the globin bands were stained with Coomassie blue and scanned by a Beckman densitometer (model CDS-200).

Isolation of Hemoglobins and Globins. Hb Portland-1 ($\zeta_2\gamma_2$) was partially purified from the hemolysates of infants with Hb Bart's hydrops fetalis syndrome, using DEAE-cellulose column chromatography with glycine/KCN/NaCl elution (9). Three main fractions were eluted. The first fraction, hereafter known as fraction 1, contained about 60% Hb Portland-1, the rest being Hb Bart's (γ_4) and Hb H (β_4).

Hb A ($\alpha_2\beta_2$) and Hb F ($\alpha_2\gamma_2$) were purified from adult cord blood hemolysates (9). α-, β-, and γ-globin chains were isolated by carboxymethyl-cellulose/urea column chromatography (7) followed by dialysis against normal saline.

Radioimmunoassay for ζ-Globin. Rabbits were repeatedly immunized with fraction 1. Antisera were obtained with a titer of 1:8 against fraction 1, as measured by the Ouchterlony double diffusion technique. Affinity chromatography using cyanogen bromide-activated Sepharose 4B (Pharmacia) coupled with normal cord blood hemolysate was used to obtain monospecific anti-ζ-globin antibodies (12). A purified IgG fraction was obtained by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography (12).

Iodination of fraction 1 with $Na^{125}I$ (New England Nuclear; 17.4 Ci/mg of iodide; 1 Ci = 37 GBq) was performed by the chloramine-T method (13). Liquid-phase radioimmunoassay was done by a previously published procedure (14). Briefly, 50 μl of test hemolysate, usually containing 2–4 μg of hemoglobin, was allowed to react with 50 μl of rabbit monospecific anti-ζ-globin IgG at 1:400 dilution for 2 hr at room temperature. All samples and standards were assayed in triplicate. The antibody titer was chosen to give 50% binding of ^{125}I-labeled fraction 1 in the absence of added unlabeled ligand (15). Fifty microliters of ^{125}I-labeled fraction 1 containing approximately 20,000 cpm was added to the mixture and incubated for a further 2 hr. Subsequently, 100 μl of goat-anti-rabbit IgG antibody (Antibodies, Inc.) was added, immediately followed by 100 μl of 7% (wt/vol) polyethylene glycol (Sigma) to give a final concentration of 2%. The tubes were spun in an Eppendorf centrifuge, the supernatant was discarded, and the radioactivity in the precipitates was determined in a Beckman 300 γ counter. The amount of ζ-globin chains present was determined from a standard curve constructed for each assay by using known amounts of unlabeled fraction 1 as the competing antigen.

RESULTS

The specificity of the rabbit IgG antibodies against ζ-globin chains was ascertained by radioimmunoassay using unlabeled fraction 1, purified Hb A, Hb F, and isolated α-, β-, and γ-globin chains as the competing antigens. The antibodies did not react with either Hb A or Hb F up to 4 μg per

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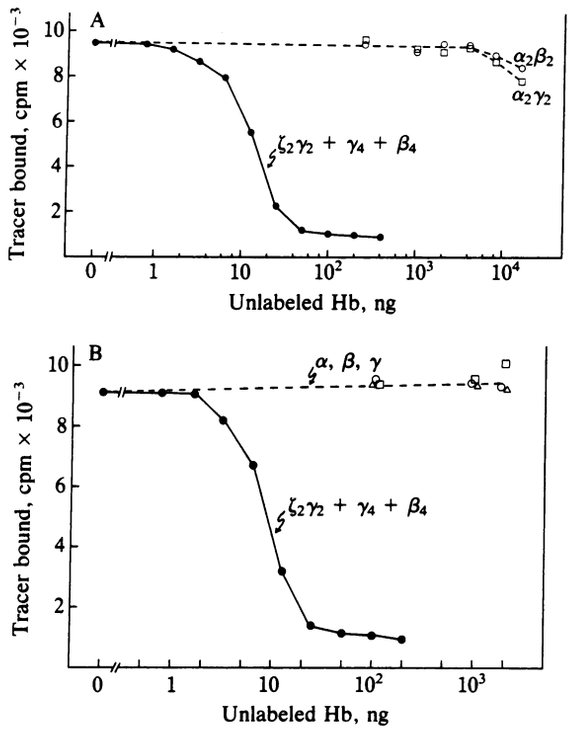

FIG. 1. Radioimmunoassay using rabbit anti-ζ-globin antibodies. The unlabeled competing antigens in these assays are fraction 1 containing $\zeta_2\gamma_2$, γ_4, and β_4 (●) in both panels and purified Hb A ($\alpha_2\beta_2$, ○) or Hb F ($\alpha_2\gamma_2$, □) in *A* or isolated α-, β-, or γ-globin chains (○, □, △) in *B*. The radioactive tracer added to each assay is ^{125}I-labeled fraction 1 containing about 20,000 cpm.

assay tube (Fig. 1*A*). Purified α-, β-, and γ-globin chains did not react with the antibodies up to 2 μg per assay tube (Fig. 1*B*). The assay can readily detect 3 ng of fraction 1 containing 1 ng of ζ-globin chains. The radioimmunoassay standard curve had the steepest slope within the range of 5–20 ng of detectable fraction 1, and the amount of hemolysate used in the assay was adjusted to give a result in this range. For each hemolysate sample, the amount of ζ-globin present was determined on at least two different occasions, each time in triplicate. The results presented here are means of these six or more determinations. To evaluate the reproducibility of the radioimmunoassay, one hemolysate sample was assayed in triplicate in 44 runs within a period of 6 months. The mean value of ζ-globin present was 0.30% (range 0.25% to 0.35%) and the between-run standard deviation was 0.025%.

Fig. 2 illustrates the globin chains present in the fraction 1

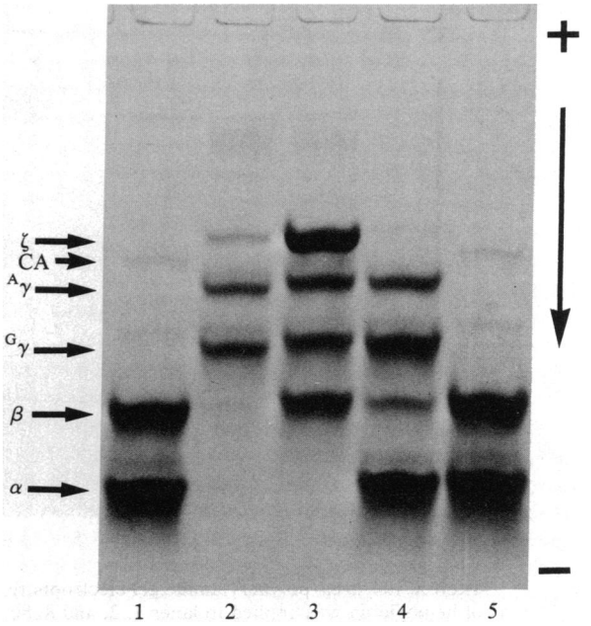

FIG. 2. Triton X-100/urea/polyacrylamide gel electrophoresis of hemolysates. Lanes: 1, adult hemolysate; 2, hemolysate of infant with Hb Bart's hydrops fetalis syndrome; 3, fraction 1; 4, normal cord blood, which was found by radioimmunoassay to contain 0.04% ζ-globin chains; 5, adult hemolysate. CA, carbonic anhydrase.

that was used as the standard in all the radioimmunoassays. ζ-Globin chains account for 32% of the globin chains present in fraction 1. The radioimmunoassay results were converted to an amount of ζ-globin chains expressed as a percentage of total hemoglobins present in the various hemolysate samples tested.

The hemolysates of seven patients with hereditary Hb H disease were studied. The hematological results are listed in Table 1. All seven patients were of Oriental origin. The putative α-globin genotype of five patients was $--/-\alpha$. Patient T.W. had Hb Q and his putative genotype was $--/-\alpha^Q$ (16). Patient S.S. had Hb Constant Spring and Hb A and, therefore, his putative genotype was $--/\alpha\alpha^{cs}$. The amount of Hb H in hemolysates from these patients varied from 3.3% to 13.1%, and ζ-globin chains as measured by radioimmunoassay ranged from 0.09% to 0.28% (Table 1). Fig. 3 shows that, on Triton X-100/urea/polyacrylamide gel electrophoresis of the hemolysates from all of these patients, there are globin bands with the same electrophoretic mobility as authentic ζ-globin chains.

The hemolysates of 24 patients with α-thalassemia trait

Table 1. Hematological data of patients with hereditary Hb H disease

Patient	Age, years	Hb, g/dl	Mean corpuscular volume, fl	Reticulocyte count, %	Hb A_2, %	Hb F, %	Hb H, %	α/β-Globin synthetic ratio	ζ-Globin chain,* %
P.S.	27	11.3	64.7	3.9	1.1	0.9	11.1	0.34	0.09
P.R.	23	10.0	63.6	7.5	1.2	0.9	6.8	0.30	0.13
H.S.	33	10.2	64.1	10.1	1.0	1.7	—†	0.51	0.15
K.C.	25	8.7	56.3	7.4	1.6	1.9	8.5	ND	0.15
A.C.	46	10.1	56.5	6.9	1.5	0.6	3.3	ND	0.22
T.W.	1	9.2	49.0	ND	—‡	—‡	—‡	ND	0.28
S.S.	3	3.4§	59.0	10.3	—§	3.5	13.1	0.55	0.22

ND, not determined.
*ζ-Globin chains were determined by radioimmunoassay.
†Starch gel electrophoresis of hemolysate revealed Hb H.
‡DEAE-cellulose chromatography of hemolysate revealed 84.7% Hb Q, 0.8% Hb Q_2, 5.9% Hb F, 8.6% Hb H and Hb Bart's.
§Child was admitted with gastroenteritis and had a history of aspirin ingestion. DEAE-cellulose chromatography of hemolysate revealed 73.8% Hb A, 1.3% Hb A_2 and Hb Constant Spring, 3.5% Hb F, 8.3% Hb A_1 and Hb F_1, and 13.1% Hb H.

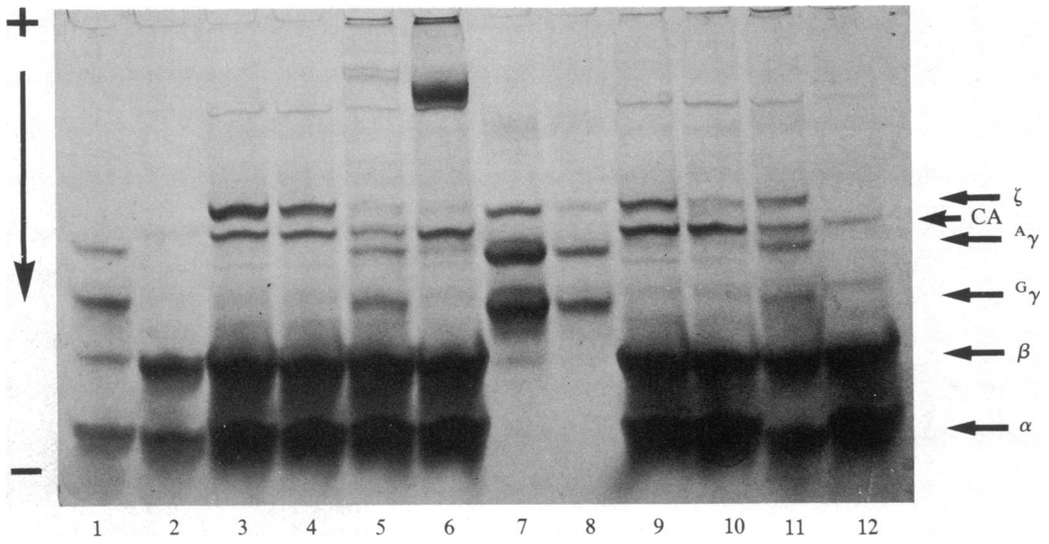

FIG. 3. Triton X-100/urea/polyacrylamide gel electrophoresis of hemolysates of the seven Hb H patients as presented in Table 1. Twenty micrograms of hemoglobin was applied to lanes 1, 2, and 8; 80 μg to lanes 7 and 12; and 120 μg to lanes 3–6 and 9–11. Lanes: 1, normal cord blood hemolysate; 2, normal adult hemolysate; 3, patient P.S.; 4, patient P.R.; 5, patient S.S.; 6, patient H.S.; 7 and 8, hemolysate of infant with Hb Bart's hydrops fetalis syndrome; 9, patient K.C.; 10, patient A.C.; 11, patient T.W.; 12, normal adult hemolysate. CA, carbonic anhydrase.

were investigated. The diagnosis of α-thalassemia trait was based on a hypochromic microcytic blood picture, without evidence of iron deficiency, and with normal Hb A_2 and Hb F levels. In addition, Hb H inclusion bodies were demonstrated in all subjects. Eight of these patients, all of Oriental origin, had clearly demonstrable ζ-globin chains by radioimmunoassay, ranging from 0.07% to 0.27% (Table 2). ζ-Globin chains were also detected by the Triton X-100/urea/polyacrylamide gel electrophoresis (data not shown). The other 16 patients, all of non-Oriental origin, did not have demonstrable ζ-globin chains by the radioimmunoassay (Table 3).

The hemolysates of another 20 patients with β-thalassemia trait were also studied and no ζ-globin chains were detected (Table 3).

DISCUSSION

The present investigation provides clear evidence that embryonic ζ-globin chains are present in children and adults with hereditary Hb H disease as well as in some patients with α-thalassemia trait. It is likely that, in these individuals, the ζ-globin chains interact with the β-globin chains to form Hb Portland-2 ($\zeta_2\beta_2$) as described recently (17).

The ζ-globin chains were quantitated by radioimmunoassay. The monospecificity of the rabbit IgG antibodies against ζ-globin chains was demonstrated by the fact that Hb A or Hb F as well as purified α-, β-, and γ-globin chains did not react significantly with the antibodies in the radioimmunoassay. Recently, we have studied three patients with acquired Hb H disease (18), and no ζ-globin chains were detected by radioimmunoassay, providing further evidence that the antibodies do not react with Hb H (β_4). The presence of ζ-globin chains in the hemolysates of those patients investigated in the present study was confirmed by Triton X-100/urea/polyacrylamide gel electrophoresis.

The mechanism for the continued expression of the embryonic ζ-globin genes in children and adults with hereditary Hb H disease and α-thalassemia trait is not known. In the β-globin gene cluster, deletion of part of the intergenic sequences between the $^{A}\gamma$- and δ-globin genes, such as in δβ-thalassemia or hereditary persistence of fetal hemoglobin, is usually associated with various degrees of augmented expression of γ-globin genes in adults. These observations lead to the hypothesis that some part of the DNA sequences between the $^{A}\gamma$- and δ-globin genes may play an important regulatory role in the expression of γ-globin genes in adults (19, 20). It is conceivable that a similar regulatory mechansim may occur in the ζ–α-globin gene complex. However, the amount of ζ-globin present in the adult α-thalassemic patients reported here is considerably less than that of γ-globin present in patients with the aforementioned hereditary disorders involving the γδβ-globin gene complex. It is of interest that the intergenic distance between the ζ- and α-globin genes is about 19 kilobase pairs, further than between any other two neighboring globin genes (21).

In 8 out of the 24 patients with α-thalassemia trait, ζ-globin chains were detected at a level similar to that found in the

Table 2. Hematological data of α-thalassemia trait patients of Oriental origin

Patient	Age, years	Hb, g/dl	Mean corpuscular volume, fl	Reticulocyte count, %	Hb A_2, %	Hb F, %	α/β-Globin synthetic ratio	ζ-Globin chain, %
H.P.	46	13.2	66.0	1.7	2.2	0.5	ND	0.07
S.W.	35	15.1	65.1	ND	2.1	0.7	ND	0.12
T.H.	25	13.8	73.6	1.7	2.3	0.7	ND	0.13
F.Y.	51	12.9	71.0	2.0	3.0	0.6	ND	0.14
S.C.	42	12.0	71.7	2.2	2.6	1.6	ND	0.15
O.S.	8	11.0	63.4	1.8	2.6	0.8	ND	0.19
J.P.	4	12.1	61.6	1.0	3.1	1.0	0.65	0.21
M.S.	18	11.3	64.5	1.0	2.3	1.2	ND	0.27

ζ-Globin chains were determined by radioimmunoassay. ND, not determined.

Table 3. Hematological data of α-thalassemia trait patients of non-Oriental origin and β-thalassemia trait patients

Diagnosis	No. of patients	Age, years	Hb, g/dl	Mean corpuscular volume, fl	Reticulocyte count, %	Hb A_2, %	Hb F, %	ζ-Globin chain, %
α-Thalassemia trait	16	22.5 ± 18.8 (1–53)	12.7 ± 1.2 (10.8–15.3)	70.0 ± 4.4 (62.7–78.0)	1.2 ± 0.4 (0.5–2.0)	2.9 ± 0.8 (1.9–5.6)*	0.8 ± 0.6 (0.4–2.4)	Undetectable
β-Thalassemia trait	20	33.6 ± 16.9 (4–72)	11.6 ± 1.2 (9.9–13.7)	64.5 ± 4.6 (57.2–74.2)	2.6 ± 1.1 (1.2–5.3)	5.5 ± 0.5 (4.3–6.1)	1.3 ± 0.8 (0.5–3.3)	Undetectable

ζ-Globin chains were determined by radioimmunoassay. Results are expressed as mean ± SD (range).
*One patient with both α- and β-thalassemia trait had 5.6% Hb A_2. Otherwise the range for Hb A_2 in the group of α-thalassemia trait patients was from 1.9 to 3.3.

other 7 patients with hereditary Hb H disease. All of these 8 patients with α-thalassemia trait are of Oriental origin, in whom α-thalassemia trait is most commonly due to deletion of two α-globin genes on one chromosome (1). All the other 16 patients in whom ζ-globin chains were not detected are of non-Oriental origin. It is conceivable that ζ-globin chains are present in these patients at a concentration below the detection limit of the assay, which is 0.03% ζ-globin chains in hemolysates. Among non-Orientals, α-thalassemia trait is usually due to deletion of one α-globin gene from each of the two homologous chromosomes, or it is due to nondeletion abnormalities (22–24). In the Hb Bart's hydrops fetalis syndrome due to the deletion of all four α-globin genes, the ζ-globin chains are present in fetuses even in the third trimester. Taken together, these observations suggest that the deletion involving two α-globin genes on the same chromosome is associated with the continued expression of ζ-globin genes in adult individuals. It should be informative to study the possible correlation between the genotypes of the ζ–α-globin gene cluster and the expression of the embryonic ζ-globin gene in α-thalassemic patients from different populations.

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