Haemoglobin Lepore in Cyprus

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Summary. Structural analysis documented the presence of haemoglobin Lepore_{Washington} (= Lepore_{Boston}) in a Greek Cypriot family and provided further evidence that, of the various types of Lepore mutants, only one is common in the Mediterranean area. Two individuals in this family were heterozygous for both Hb Lepore and β thalassaemia, but they exhibited striking differences in the clinical severity and course of the disease. The data illustrate that additional environmental or genetic factors play roles in determining or modifying the pathophysiological consequences of highly specific molecular defects and, thus, their ultimate clinical phenotypes.

Since the original description of Hb Lepore in persons of Mediterranean descent (Gerald and Diamond, 1958), several studies have shown that the Lepore mutants are not uncommon in southern European and near Eastern populations. The Hb Lepore trait has been detected among Greeks (Fessas *et al*, 1962; Malmos *et al*, 1962; Stamatoyannopoulos and Fessas, 1963, 1964; Fraser *et al*, 1964; Stamatoyannopoulos, unpublished data), Yugoslavians (Fraser *et al*, 1966; Duma *et al*, 1968), Italians (Pearson *et al*, 1959; Silvestroni and Bianco, 1963; Barkham *et al*, 1964; Labie *et al*, 1966; Quattrin *et al*, 1967; Quattrin and Ventruato, 1974), Rumanians (Rowley *et al*, 1969), Turkish Cypriots (Beaven *et al*, 1964), and Iranians (Rahbar *et al*, 1974). The data suggest that the Lepore trait is spread throughout the areas in which the β -thalassaemia genes exist at polymorphic frequencies. In this paper we present evidence that the Lepore trait is present in Greek Cypriots. Structural analysis identified the variant observed in Cyprus as Hb Lepore_{Washington} (= Lepore_{Boston}) and supports the view that only one, among several possible Lepore mutants, is common in Mediterranean populations.

Methods

Haematological studies were performed using standard techniques. Haemolysates were examined by starch-gel electrophoresis in a Tris-borate buffer system, pH 8.6 (O. Smithies, cited in Huehns and Shooter, 1965). Levels of Hb F were measured by alkali denaturation

(Bette *et al*, 1959). In addition, the relative proportions of haemoglobins A, A₂, F, and the abnormal component were determined following their isolation by chromatography of 2.5 × 44.5 cm columns of DEAE-Sephadex (A-50) (Huisman and Dozy, 1965), employing a gradient formed by mixing 1 litre of 0.05 mol/l Tris-HCl, pH 8.0 (starting buffer) with 1 litre of 0.05 mol/l Tris-HCl, pH 7.3 (limiting buffer). All buffers were 0.002 mol/l in KCN. Isolated haemoglobins were identified and checked for purity by starch-gel electrophoresis as described above.

Following acid-acetone precipitation of haem-free globin from the abnormal chromatographic fraction, α and non- α chains were separated on a 2 × 10 cm column of Whatman CM-52 carboxymethyl-cellulose according to the method of Clegg *et al* (1968).

Isolated non- α chains (56 mg) were dialysed free of salts, lyophilized, and aminoethylated as described by Jones (1964), with the exceptions that urea was replaced by an equimolar amount of guanidine-HCl and 2-mercaptoethanol was substituted by 40 mg of dithioerythritol. After digestion by trypsin-TPCK (Worthington Biochemical Co), 2.8 mg of peptides were subjected to paper electrophoresis in a buffer of pyridine, acetic acid, and water (10:0.4:90 by volume), pH 6.4 (Ingram, 1958), followed by descending chromatography in a solvent of pyridine, isoamyl alcohol, and water (35:35:30 by volume) (Baglioni, 1965). Peptides were located by staining with 0.3% buffered ninhydrin in acetone and tryptophan-containing peptides were detected with the Ehrlich reagent (Easley, 1965). A second map was

Received 15 August 1974.

prepared using 5.9 mg of the digest. Selected peptides were eluted (Sanger and Tuppy, 1951; Clegg *et al.*, 1966) with 6 mol/l HCl, hydrolysed for 24 h in sealed capillary tubes, and subjected to amino-acid analysis.

The ratio of γ chains containing glycine in position 136 to those with alanine in this position was determined for Hb F from the *proposita*. Haem-free globulin F was cleaved by cyanogen bromide and the C-terminal γ -chain fragment (γ CB-3) was isolated on a 1.8×292 cm column of Sephadex G-50 (fine) equilibrated and developed with 1% formic acid (Nute *et al.*, 1973). Following recovery by lyophilization, the γ CB-3 fragments were dissolved in 6 mol/l HCl and duplicate hydrolyses were made *in vacuo* at 108°C for 24 h before amino-acid analysis.

Family report

Proposita. The *proposita* (II.1, Fig. 1) was referred for study at the age of 8 because she presented with pallor and her spleen was palpable to 3 cm below the costal margin. Her bodily development was normal. Haematological examination revealed a moderate anaemia (Hb = 7.8 g/dl, PCV = 33, RBC = $4.7 \times 10^{12}/\text{l}$, MCV = 70 fl, MCH = 16.6 pg, and MCHC = 23.7 g/dl). Blood films showed striking abnormalities in erythrocyte morphology, with hypochromia, moderate microcytosis and anisocytosis, poikilocytosis, leptocytosis, mild anisochromia, moderate numbers of target cells, and a few fragmented cells. The reticulocyte count was 2.1% and no normoblasts were observed. Chromatography of the *proposita*'s haemoglobins (Fig. 2) and identification of the various components by starch-gel electrophoresis showed that Hbs F + F₁ + A₃ constituted 79.5% of the total. Haemoglobins A and A₂ were present at levels of 10.5% and 2.9%, respectively; the remaining 7.5% consisted of an abnormal component which resembled the Hbs Lepore both electrophoretically and chromatographically. The *proposita* had never required transfusions.

Other family members. Examination of other members of the family (Fig. 1) revealed that the father had typical β -thalassaemia trait with elevated Hb A₂ (5.3%). The mother showed haematological signs of thalassaemia trait (Hb = 12.4 g/dl, PCV = 41, RBC = 5.61

FIG. 2. Elution profiles produced by chromatography of haemoglobins from the *proposita* and her mother on DEAE-Sephadex (see text for details).

$\times 10^{12}/\text{l}$, MCV = 73 fl, MCH = 22 pg), and chromatography of her haemoglobins (Fig. 2) revealed the presence of an abnormal component (constituting 11.1% of the total Hb) with the same properties as that of the *proposita*. Her Hb A₂ level was normal (2.3%) and Hb F was elevated as judged both by chromatography (Hbs F + F₁ + A₃ = 7.1%) and alkali denaturation (Hbs F + F₁ = 2.8%).

One of the *proposita*'s sibs (II.2) had β -thalassaemia trait (Hb A₂ = 4.6%, Hb F = 3.2%). The second sib (II.3) showed the clinical and haematological manifestations of thalassaemia major, presenting with severe anaemia in early infancy; she has received transfusions since the age of 6 months and had undergone splenectomy at 4 years of age. Monthly transfusions are required for the management of her anaemia. At the time the family study was conducted, the Hb level in this child was 4 g/dl, and haemoglobin electrophoresis showed large amounts of Hb A (over 85% of the total) derived from the transfused blood.

Results

Peptide maps (Fig. 3) of the non- α chains from the abnormal haemoglobin showed no sign of a peptide corresponding in position to β T₃. However, a peptide was present whose position closely

FIG. 1. Family pedigree.

FIG. 3. Tracing of map produced by paper electrophoresis and chromatography of tryptic peptides from the δ - β chains of the proposita (see text for details).

approximated that predicted for δT_3 . Moreover, the peptides whose locations roughly approximated those of βT_2 and βT_{10} appeared to have slightly slower chromatographic mobilities than would be expected of normal β -chain peptides, suggesting that the abnormal chain was δ -like through residues 86 or 87. The presence of a peptide corresponding in position to βT_{12b} indicated that the chain was β -like in structure from positions 116 through 146.

Amino-acid analyses (Table I) confirmed the suspected δ - β structure of the abnormal chain,

showing it to be δ -like from the N-terminus through position 87 and β -like in positions 116 through the C-terminus. Positions 88 through 115 are identical in both δ and β chains and, hence, could be specified by the corresponding portions of either the β - or δ -chain genes. Analysis of peptides T_{10} , T_{11} , T_{12a} , and T_{12b} (constituting residues 83 through 120) produced no evidence of deletion or duplication of any residues, indicating that the crossover which led to the formation of the δ - β gene resulted in the synthesis of a chain with 146 residues. Thus, the structure of the abnormal chain corresponds to that described for the δ - β chain of Hb Lepore^{Washington} (= Lepore^{Boston}) (Baglioni, 1962, 1965; Baglioni and Ventruto, 1968).

The results of γ CB-3 analysis indicated that the proportions $\alpha\gamma$ and $\Lambda\gamma$ chains in the Hb F of the proposita are well within normal limits. The values of 0.41 and 0.40 residues of glycine and 2.63 residues of alanine obtained from the two analyses demonstrate that the ratio of $\alpha\gamma$ to $\Lambda\gamma$ chains is 2:3, the ratio expected for the Hb F of normal individuals after the first year of life (Schroeder and Huisman, 1970). Apparently, the deletion of portions of the δ - and β -chain genes that occurs with the formation of the δ - β gene of Hb Lepore^{Washington} has no obvious effects on the relative activities of the $\alpha\gamma$ and $\Lambda\gamma$ genes in the genome.

Discussion

The structures of the Lepore haemoglobins as products of unequal crossing over between β and δ

TABLE I
AMINO-ACID COMPOSITIONS OF SELECTED PEPTIDES FROM THE δ - β CHAINS OF
Hb LEPORE^{WASHINGTON}*

Peptide	δ -like				Identical in β and δ		β -like	
	T_2	T_3	T_5	T_{10}	T_{11}	T_{12a}	T_{12b}	T_{13}
Asp	0.94 (1)	1.86 (2)	2.88 (3)	1.07 (1)	1.96 (2)	1.05 (1)		0.98 (1)
Thr	0.84 (1)			0.97 (1)				
Ser			2.81 (3)	1.87 (2)				
Glu		1.00 (1)	1.12 (1)	1.91 (2)	1.00 (1)			3.00 (3)
Pro			1.82 (2)		0.95 (1)			1.91 (2)
Gly	1.01 (1)	2.98 (3)	2.07 (2)	1.01 (1)		1.01 (1)	1.02 (1)	
Ala	2.09 (2)	2.03 (2)	1.21 (1)				1.06 (1)	2.21 (2)
Val	1.05 (1)	3.01 (3)	1.10 (1)		0.97 (1)	2.01 (2)	0.95 (1)	0.97 (1)
Met			0.83 (1)					
Leu	1.00 (1)	1.06 (1)	0.92 (1)	1.94 (2)	0.83 (1)	3.00 (3)	0.95 (1)	
Tyr								0.76 (1)
Phe			2.79 (3)	1.00 (1)	1.01 (1)			1.01 (1)
Lys	1.00 (1)		1.02 (1)	0.98 (1)			1.00 (1)	0.91 (1)
His				1.00 (1)	0.89 (1)			
Arg					0.94 (1)			
Trp†	+	1.00 (1)						
SAE-Cys				0.87 (1)		0.75 (1)		

* Those peptides in which β and δ chains are known to differ, plus peptides constituting the region in which the transition from δ - to β -chain structure occurs (residues 88 to 115), were selected for analysis. Numbers in parentheses refer to the compositions of corresponding peptides in normal δ (for the δ -like peptides) and β (for the β -like peptides) chains.

† Detected on maps after staining with the Ehrlich reagent (Easley, 1965).

Fig. 4. The three structural types of δ - β chains found in the Lepore haemoglobins: Hb Lepore^{Hollandia} (Barnabas and Muller, 1962), Hb Lepore^{Baltimore} (Ostertag and Smith, 1969), and Hb Lepore^{Washington} (Baglioni, 1962; Labie *et al.*, 1966). The broken lines connecting the δ - and β -like sequences represent those segments of the chains that are identical in both δ and β chains.

genes have been well documented. Of the several theoretical δ - β products of such events (Smithies, 1964; Stamatoiyannopoulos *et al.*, 1969), three have been characterized (Fig. 4). Analyses of the Lepore mutants from Italian (Baglioni, 1965; Labie *et al.*, 1966; Baglioni and Ventruto, 1968; Quattrin and Ventruto, 1974) and Greek (Baglioni and Ventruto, 1968) individuals suggest that only one type, Hb Lepore^{Washington}, is common among Mediterranean populations. Our study supports this view, as it demonstrates unequivocally that the abnormal haemoglobin in the Cypriot family is identical in structure to Hb Lepore^{Washington}. The uniformity of the structural analyses suggests that the Lepore variants in the various Mediterranean populations share a common origin or that the type of crossover giving rise to the δ - β chains of Hb Lepore^{Washington} occurs more frequently than do those responsible for other Lepore mutants. Alternatively, if one assumes that all types of Hb Lepore occur as new mutations with nearly equal frequencies, the Hb Lepore^{Washington} trait may confer a greater selective advantage on its carriers than either the Lepore^{Baltimore} or Lepore^{Hollandia} traits, especially in those environments where the thalassaemia trait appears to be favoured by selection.

The discordance in the clinical courses of II.1 and II.3 described in this report is of interest. Both have the Hb Lepore/ β -thalassaemia genotype and, presumably, identical defects in β -chain synthesis. The older child has a mild disease which was first detected at the age of 8 years; the clinical course is that of thalassaemia intermedia. The younger child has shown the signs of a severe β -thalassaemia major; typically, she began to receive transfusions at the age of 6 months. This striking difference illustrates that several genetic and/or environmental factors, in addition to the manifestations of the basic molecular lesion, take part in shaping the final clinical presentation of a genetic disease. In the case of these two sibs, differences in the proportion of the erythroblastic population involved in γ -chain

synthesis, or differences in the rate of degradation of excess α chains in normoblasts might underlie the discordance in clinical phenotypes.

Ashiotis *et al.* (1973) have shown that 10% of the Cypriot population carry α -thalassaemia-1 or α -thalassaemia-2 genes. Given this high frequency of carriers, it is not unlikely that the least severely affected sib (II.1) bears an α -thalassaemia gene in addition to being heterozygous for both Hb Lepore and β thalassaemia. Association of α thalassaemia with homozygosity for the β -thalassaemia determinant is expected to ameliorate the effects of the latter condition by partially correcting the imbalance between amounts of α and β chains synthesized (Fessas, 1967; Kan and Nathan, 1970). Reduction in the level of excess α chains will lower the extent of ineffective erythropoiesis and produce a more favourable clinical picture (Fessas, 1967). Thus, approximately 1 in 10 Cypriots with homozygous β thalassaemia should present with mild symptoms, owing to the presence of an α -thalassaemia determinant in his genome; this is the approximate proportion of mildly affected β -thalassaemia homozygotes in the patient population of Cyprus and among Cypriots attending paediatric clinics in London (B. Modell, personal communication). It appears likely that the inheritance of an α -thalassaemia gene by II.1 but not by II.3 is the cause of the clinical discordance between the two sibs of identical Hb Lepore/ β -thalassaemia genotypes. Unfortunately, we have not yet been able to test this possibility by carrying out studies of haemoglobin synthesis.

This research was supported by grants GM15253 and RR00166 from the National Institute of Health, US Public Health Service. We thank Ms Sigrid Hornung for expert technical assistance.

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