Homozygous haemoglobin D Punjab

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Summary. A homozygote for the gene controlling Hb D Punjab is described. The diagnosis is supported by the peptide analysis of the haemoglobin and the examination of both parents. There was no anaemia or reticulocytosis and there was balanced synthesis of both the α^A - and β^D -globin chains in reticulocytes. However, the oxygen affinity of the haemolysate had a small but significantly higher oxygen affinity than normal. The only other abnormalities were a somewhat decreased osmotic fragility and a slight anisocytosis of the red cells.

Haemoglobin D was discovered in 1951 by Itano as an adult haemoglobin which had the same electrophoretic mobility as Hb S, but did not cause the sickling phenomenon, and of which the deoxyhaemoglobin had the solubility of deoxyhaemoglobin A rather than that of deoxyhaemoglobin S. When the tryptic peptides of different examples of Hb D were compared, it was seen that they did not all carry their chemical change in the same part of the haemoglobin molecule (Benzer *et al*, 1958).

In 1959, Ingram traced the change in abnormal haemoglobins to either the α or the β chains by making fingerprints of the isolated haemoglobin. An alternative method to achieve this was hybridization of different haemoglobins by dissociation followed by recombination (Singer and Itano, 1958). With these methods it could be established that 'Hb D' could be either an α -chain or a β -chain variant. Baglioni examined, in 1962, five haemoglobins D of widely differing ethnic origin which all had an amino-acid substitution at position 121 of the β chain where the usual glutamic acid residue was replaced by one of glutamine. These specimens included one from Punjab where 3% of the population had been found to be heterozygous carriers of this variant and the variant was named Hb D Punjab. Later the original Hb D of Itano (1958) was found by Babin *et al* (1964) to be the same variant

and therefore the description of Hb D Los Angeles is often used.

It is of interest that very few descriptions of homozygotes for the gene responsible for Hb D Punjab have been published and of these only one was backed up by family study (Dutta *et al*, 1972). The heterozygous state was found in both parents of a 21-year-old Sikh from the Ferozepore District of the Punjab. A family study is important because the heterozygote for the genes controlling Hb D Punjab and β^0 thalassaemia shows Hb D as the only adult haemoglobin. As, however, 'haemoglobin D' comprises a large heterogeneous group of β -chain variants with similar electrophoretic properties, a full substantiation of the homozygous state for the genes controlling Hb D Punjab should ideally be supported by the demonstration of the peptide abnormality which in the case of this haemoglobin can be provided by simple fingerprinting.

This paper describes a homozygote for the genes responsible for Hb D Punjab.

Methods

Haemoglobin electrophoresis was carried out on paper (pH 8.9, for details see Lehmann and Huntsman, 1966), cellulose acetate (pH 8.9, Marengo-Rowe, 1965), and agar gel (pH 6.2, Robinson *et al*, 1957).

Fingerprints and peptide analysis followed those summarized by Sick *et al* (1967).

Separation of α^A and β^D chains was performed by the method of Clegg *et al* (1966).

Haematological parameters were obtained by a Coulter 'S' Autoanalyzer.

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Osmotic fragility test was carried out according to the method described by Dacie and Lewis (1968). Autohaemolysis test was performed by the method of Grimes *et al* (1968).

The biosynthesis was carried out as follows. Blood was freed from plasma by centrifugation and washed three times with isotonic saline solution (Lingrel and Borsook, 1968). A reticulocyte-rich preparation of red cells was prepared (White *et al*, 1971) and 1 ml of packed cells was pre-incubated for 15 min at 37°C in the medium described by Lingrel and Borsook (1968). 100 μ Ci 3 H-leucine (38 Ci/mmol) was added and aliquots removed after 20, 40, and 60 min incubation into 100-fold excess of ice-cold isotonic saline. The cells were washed free from excess isotope with ice-cold isotonic saline (3 $\times$). The cells were then haemolysed by the addition of distilled water and globin prepared directly from the haemolysate by precipitation from acidified acetone at -20°C (1.5% concentrated HCl in acetone). The α^A - and β^D -globin chains were separated by ion exchange chromatography on Whatman CM 23 cellulose as described by Clegg *et al* (1966). The fractions corresponding to each globin chain were pooled and the total volumes estimated. Aliquots, 1 ml, were removed to estimate the total incorporation of 3 H-leucine into globin (total counts per min, cpm). The remainder was dialysed overnight against 2 $\times$ 10l of 0.5% formic acid at 4°C. The specific activities of the globin chains in cpm 3 H-leucine per OD 280 nm were obtained from measurements carried out on the dialysates. 3 H-leucine determinations were performed in a 'Tracerlab Coru Matic 200' liquid scintillation spectrometer using the scintillator triton $\times$ 100, toluene, PPO (Hunt *et al*, 1968).

Oxygen affinity measurements were performed as follows. Haemolysates were prepared from the blood of the propositus and a normal control by extraction with carbon tetrachloride. The Hb concentrations were adjusted to 0.1% with 0.1 mol/l potassium phosphate buffer containing 0.5 mmol/l EDTA. Oxygen affinity data were recorded by the automated method of Imai *et al* (1970). Measurements were made at 20° at a wavelength 474 nm. The methaemoglobin concentrations were determined before and after each recording from the value of absorbancies at 576 nm and 508 nm.

Results

The subject is a citizen of India living in London who became a regular blood donor at a London

Blood Transfusion Centre. Table I lists the haematological findings made in this donor and his parents. The blood picture was fully normal in both parents, but in the propositus there was some degree of anisocytosis and some target cells could be seen (Fig. 1). However, these were very occasional and might not have been noticed in an ordinary routine investigation. He was discovered in the course of a screening programme when the haemoglobin of immigrants were routinely examined by cellulose acetate electrophoresis at pH 8.9. The electrophoretic pattern showed no haemoglobin band in the position of Hb A but only in that of Hb S or D.

FIG. 1. The appearance of the propositus' red cells. Note the occasional target cell and slight anisocytosis ($\times$ 600).

TABLE I
HAEMATOLOGICAL FINDINGS OF THE PROPOSITUS AND HIS PARENTS

	Hb (g/dl)	RBC ($\times 10^{12}/l$)	PCV	MCH (pg)	MCHC (g/dl)	MCV (fl)	Reticulocytes (%)	Hb F (%)	Hb A ₂ (%)
Propositus	15	5.59	44	27	34	79	1.4	0.2	3.3
Father	15	—	46	33	33	100	1.2	0.7	
Mother	13.2	—	39	27	34	80	1.2	0.7

[FIG. 2. Fingerprint of the haemolysate showing the features of Hb D Punjab. Electrophoresis pH 6.4. * indicates point of application.

The sickle test and Itano's solubility test excluded the presence of Hb S. Electrophoresis on paper gave results identical to those obtained with cellulose acetate, but agar gel electrophoresis at pH 6.2 showed the haemoglobin to move in the manner of Hb A rather than that of Hb S. This is the property of most haemoglobins D and notably that of haemoglobin D Punjab. To confirm that the haemoglobin was indeed Hb D Punjab, a fingerprint was prepared and the result is shown in Fig. 2. The tryptic peptide (Tp) β^A XIII (β 121→132) which stains positive for tyrosine is absent and a new more positively charged peptide adjoining α Tp X-XI is present which stains for tyrosine.

For amino-acid analysis the α and β chains were separated and β^A Tp XIII was obtained from a fingerprint of the isolated β^A chain. The analysis was identical with that of β^A Tp XIII (Table II). However, the electrophoretic mobility of the haemoglobin and of the peptide β^A XIII indicate that there has occurred a loss of a negative charge or that an additional positive charge has been acquired. In view of the amino-acid analysis this can only be a replacement of β 121 glutamic acid by glutamine. Glutamine is converted to glutamic acid during the hydrolysis of the peptide which precedes the amino-acid analysis.

There was no evidence of thalassaemia. The MCV and MCHC were fully normal (Table I) and Hb A₂ (3.3%) was within normal limits (2.5–3.5%) and Hb F estimated according to Betke *et al* (1959) amounted to 0.2% (normal range 0.8%). Figs. 3

TABLE II
AMINO-ACID COMPOSITION OF
 β Tp XIII FROM Hb D PUNJAB
AFTER ACID HYDROLYSIS

Amino Acid	nmol	Molar Ratio
Thr	304	1.0
Glu	984	3.1
Pro	614	2.0
Ala	632	2.0
Val	340	1.1
Tyr	245	0.8
Phe	340	1.1
Lys	287	0.9

FIG. 3. Paper electrophoretic pattern (pH 8.9) of the haemoglobin of the propositus compared with that of a patient with Hb D Punjab β^0 thalassaemia. The latter differs by having a raised Hb A₂ level.

FIG. 4. Paper electrophoretic pattern of the haemoglobin of the propositus compared with that of a heterozygote for β^0 thalassaemia.

and 4 show the electrophoretic pattern of the propositus haemoglobin compared (1) with that of a patient with Hb D β^0 -thalassaemia heterozygote who also has a raised proportion of Hb A₂ and (2) with that of a β -thalassaemia heterozygote who also has a raised Hb A₂.

To complete the diagnosis of homozygous Hb D 'disease' it was necessary to examine the propositus' family. He was married but there were no children. However, it was possible to obtain the blood of both parents from India. Both were Hindus, born in Hyderabad in Sind. The grandparents had all been born in the Punjab and had moved to Sind.

Both parents were carriers of Hb D trait (Fig. 5). Haematological details are given in Table I. The only finding of note was that the father but not the mother nor her son were G6PD deficient.

FIG. 5. The haemoglobin pattern of the two parents. Both are Hb D heterozygotes. Cellulose acetate electrophoresis.

FIG. 6. Osmotic fragility.

The question arises whether there is any evidence for a Hb D 'disease' in the propositus. There was no anaemia, indeed, the subject is a regular blood donor and the reticulocyte count is within the normal range.

The serum bilirubin level was normal (10.3 μ mol/l [0.6 mg/100ml] of plasma). The liver and spleen were not enlarged. No abnormal findings were seen in the urine.

There were, however, two observations which diverged from the otherwise normal picture. The osmotic fragility of the red cells was slightly decreased. Fig. 6 shows the fragility of fresh cells

TABLE III

IN-VITRO INCORPORATION OF 3 H-LEUCINE INTO THE α^A - AND β^D -GLOBIN CHAINS OF Hb D PUNJAB

Incubation Time (min)	Total cpm		Specific Activity (cpm/OD 280)		Ratio $\alpha^A\beta^D$	
	α^A	β^D	α^A	β^D	Total Cpm	SA
60	53 250	48 430	2213	2315	1.09	0.96
40	23 900	22 890	1258	1128	1.04	1.12
20	17 390	16 590	666	621	1.05	1.07

TABLE IV

OXYGEN AFFINITY MEASUREMENTS ON HAEMOLYSATES FROM THE PROPOSITUS

Sample	pH	P ₅₀	log ₁₀ P ₅₀	n	% Met Hb
Control	6.000	12.7	1.104	3.1	2.0→7.7
Propositus	5.990	11.7	1.068	3.1	2.0→12.4
Control	6.390	13.0	1.114	3.0	2.0→4.4
Propositus	6.390	12.2	1.086	2.9	2.0→6.7
Control	7.021	9.2	0.964	3.05	1.6→5.0
Propositus	7.011	9.1	0.959	3.2	2.9→4.8
Control	7.405	5.5	0.740	3.1	1.7→3.4
Propositus	7.401	5.1	0.708	3.0	0.0→3.7
Control	7.893	2.6	0.422	3.1	1.0→2.4
Propositus	7.910	2.5	0.398	3.1	2.0→3.6

and of cells incubated for 24 h at 37° C. When haemolysis after 48 h incubation was determined with and without glucose, the results were again not normal. Haemolysis without glucose amounted to 7.0% (normal range 0.2–4.0%) and, with glucose, was 0.55% (normal range 0.0–0.9%).

Table III shows the *in-vitro* biosynthesis of globin chains in reticulocytes from peripheral blood and indicates that the synthesis of the α^A and β^D chains is balanced throughout the period of incubation.

The haemoglobin solution prepared directly from the haemolysate had a slightly higher oxygen affinity than a normal control (Table IV). The values of P_{50} were on average 94% of the corresponding control values. This indicates a small but definite increase in O_2 affinity. The cooperativity or allosteric interaction as measured by n , the exponent of the Hill equation (1910) was normal, as was the alkaline Bohr effect.

Discussion

Having established the homozygous state for haemoglobin D Punjab in the propositus, one wonders why there are so few fully documented reports. Presumably the absence of anaemia and of any notable pathological symptoms and signs prevents the homozygote coming to the notice of clinical investigators. There is some association between the proportion of a haemoglobin variant in the heterozygote and the degree of severity of a haemoglobinopathy in the homozygote (Lehmann *et al*, 1964). Many, though not all, heterozygotes for the genes for haemoglobins A and D possess an almost equal proportion of these two haemoglobins. Thus, it can be expected that homozygotes will usually be found only in the course of surveys or when they are admitted to hospital for a reason not associated with their haemoglobin variant. Nevertheless, our propositus had some slight abnormality of the red cell osmotic fragility and it would be of interest to see whether homozygotes for Hb D Punjab show an unusual severe response to stress on their haemopoiesis, for example to an infection, or in the case of women, to pregnancy. All such reports should, of course, be carefully screened for exclusion of Hb D β^0 thalassaemia.

Since the MCV and MCH of the propositus (Table I) are normal, the balanced synthesis of the α^A and β^D chains suggests that the point mutation Glu→Gln at position 121 in the β^A chain alters neither the rate nor the extent of the synthesis of the β chain. Balanced synthesis in the reticulocytes of the Hb D Punjab homozygote reported compares with other reports of balanced synthesis in homo-

zygotes for Hb S (C. Natas, personal communication) and Hb E (D. Labie, personal communication).

The increased oxygen affinity of the haemolysate from the propositus is consistent with a previous report by Huisman *et al* (1963) of a slightly increased oxygen affinity of a chromatographically purified preparation of Hb D Punjab. The present report in substantiating the earlier result rules out the possibility mentioned by Huisman *et al* (1963), that the increased oxygen affinity of their purified Hb D Punjab was possibly arising artefactually from the purification procedures.

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