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Haemoglobin F Malaysia: $\alpha_2\gamma_2$ 1(NA1) Glycine→Cysteine; 136 Glycine

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Summary. Structural studies of an abnormal haemoglobin found in a Chinese newborn but not in her parents, showed it to be a new γ-chain variant, Hb F Malaysia.

The first residue of the γ-chain was Cys and not Gly, and the γ-chain was of the type in which residue 136 is Gly and not Ala.

During a survey in Kuala Lumpur of more than 4000 cord blood samples from different racial groups for abnormal haemoglobins, one from a Chinese baby was found to have a haemoglobin component with a higher electrophoretic mobility than Hb A on starch gel electrophoresis at pH 8·6. It just separated from Hb A but moved more slowly than Hb Barts. Neither parent had the abnormal component, and it was therefore thought to be a fetal haemoglobin variant. Blood was obtained again from the baby when she was one week old, and structural studies were carried out. In this communication we report the results.

Materials and Methods

Blood was freed from plasma by centrifugation, and the red cells were washed three times with 0·9% sodium chloride solution and haemolysed, as described by Lehmann and Huntsman (1966). Paper electrophoresis of haemolysates was carried out according to the method of Cradock-Watson, Fenton, and Lehmann (1959), cellulose-acetate electrophoresis according to Marengo-Rowe (1965), and starch-gel electrophoresis according to Smithies (1959), using Tris-EDTA-boric acid buffer (pH 8·6 and 8·0) and discontinuous Tris-boric acid buffer (pH 9·5). Haemoglobin fractions were separated by chromatography on a column of DEAE Sephadex according to Huisman and Dozy (1965).

Globin was prepared by adding 10% haemoglobin solutions dropwise to 15 volumes of concentrated HCl in acetone (1·5% v/v) at −20°C. After centrifugation the supernatant containing haem was decanted, and the precipitated globin was washed four times with acetone at −20° C and dried in a stream of nitrogen.

Separation of globin chains from each of the haemoglobin fractions was done on carboxymethyl cellulose columns according to the method of Clegg, Naughton, and Weatherall (1966), using a linear gradient from 300 ml. each of 5 mM and 30 mM $Na_2HPO_4.2H_2O$ in 8 M urea containing 50 mM 2-mercaptoethanol. The chains were aminoethylated (using the modified method of Jonxis and Huisman [1968]), dialysed against 0·5% formic acid, and recovered by lyophilization.

Tryptic digests of aminoethylated chains, high voltage paper electrophoresis, and peptide mapping were done by the methods quoted by Sick *et al* (1967). The nomenclature of Gerald and Ingram (1961) is used to describe tryptic peptides. Paper chromatograms of peptic peptides from the insoluble tryptic peptides were made according to the method of Lorkin *et al* (1970). Diagnostic fingerprints were stained for tryptophan (Smith, 1953), histidine (Dalgliesh, 1952), arginine (Jepson and Smith, 1953), tyrosine (Jepson and Smith 1953), and methionine (Toennies and Kolb, 1951). Peptides from preparative fingerprints were hydrolysed in constantly boiling 6 N HCl for 24 hr at 108° C. The hydrolysates were analysed on a Locarte Amino-Acid Analyser.

The aminoethylated γ-chains were dansylated according to Gray and Hartley (1963) as described by Gray (1967). After hydrolysis, the dansyl derivatives were purified by high voltage electrophoresis at pH 4·4 and identified on polyamide layer plates as described by Woods and Wang (1967).

Sources of Chemicals. Urea ANALAR Grade was purchased from British Drug Houses. It was further purified by passing 8 M solutions through a mixing bed deionizing resin before use.

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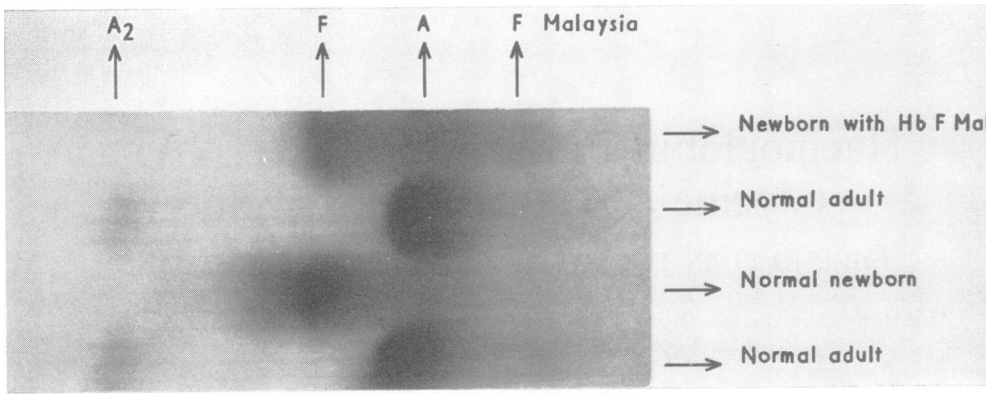

FIG. 1. Starch-gel electrophoresis in Tris-EDTA-borica cid buffer, pH 8·6, showing the abnormal haemoglobin pattern of the newborn compared with the patterns of a normal adult and a normal newborn. The haemolysate of the normal newborn was several days old and contained some methaemoglobin (benzidine stain).

Ethylenimine from L. Light and Co, and 2-mercaptoethanol from Eastman Kodak Ltd, London, were used without further purification.

TPCK-treated trypsin and pepsin were obtained from Worthington Biochemicals. Ninhydrin was manufactured by E. Merck, Darmstadt, Federal Republic of Germany.

Results

The haemolysate from cord blood showed a small amount of haemoglobin moving ahead of Hb A on electrophoresis on starch gel, cellulose acetate and paper (Fig. 1). The fraction, however, separated only little from Hb A by these methods. The abnormal component was not present in the haemolysates of either parent.

On cellulose acetate electrophoresis the abnormal component amounted to 12·8% and Hb A_2 to 0·4% of the total haemoglobin, the rest being Hb A and Hb F. Alkali denaturation of haemoglobin by the method of Singer, Chernoff, and Singer (1951) showed the Hb F to be 58·0% of the total haemoglobin. The abnormal component comprises therefore, 18·8% of the fetal haemoglobin.

On column chromatography using DEAE Sephadex, five fractions were separated (Fig. 2). Fraction 1 was in the position of Hb A, fraction 2 in that of Hb F (presumably also including some Hb A_3), and fraction 3 in that of Hb F_3. There were also two fractions (4 and 5) corresponding to what presumably were a variant Hb F and a variant Hb F_3.

It was realized that this was an unusual Hb F variant because of the poor separation on electrophoresis. Rather than further purifying the fraction obtained on chromatography, it was decided to use the individual fractions as they were and concentrate them by vacuum dialysis. This would avoid the inevitable loss of material experienced on repeated purification by paper electrophoresis and elution; and it was hoped that chain separation would succeed in isolating the variant γ-chain in an adequately purified state. As it turned out, fraction 1 showed only α^A and β^A chains, and fractions 2 and 3 only α^A and γ^F chains; but the other two fractions showed α chains, some chains in the position of γ^F, and a larger proportion of γ-chains with an increased negative charge. The normal and the variant γ-chains were running so close to each other that on their elution and subsequent peptide analysis—as will be described—some normal γ-chains were still present in the preparation of γ-chains from the variant Hb F (fraction 4), though not in that from the variant Hb F_3 (fraction 5). The proportion of normal γ-chain in fraction 4 judging from the tracing of the chain separation (subsequently pooled) was approximately 18%.

Analysis of Variant Hb F_3 (Fraction 5). The aminoethylated γ-chain from fraction 5 was digested with trypsin. The soluble tryptic peptides were fingerprinted. They showed two differences from those of the normal aminoethylated γ^F. The peptide γ^FTpI–II (residue 1–17), which gives a positive reaction for tryptophan, was absent. On a fingerprint of a normal γ-chain at pH 6·4, this peptide overlaps γ^FTpXI (residue 96–104); if the spot is cut out and re-run at pH 3·5, the two peptides, present in almost equal amounts, separate. Even before this separation, this spot in a normal γ-chain

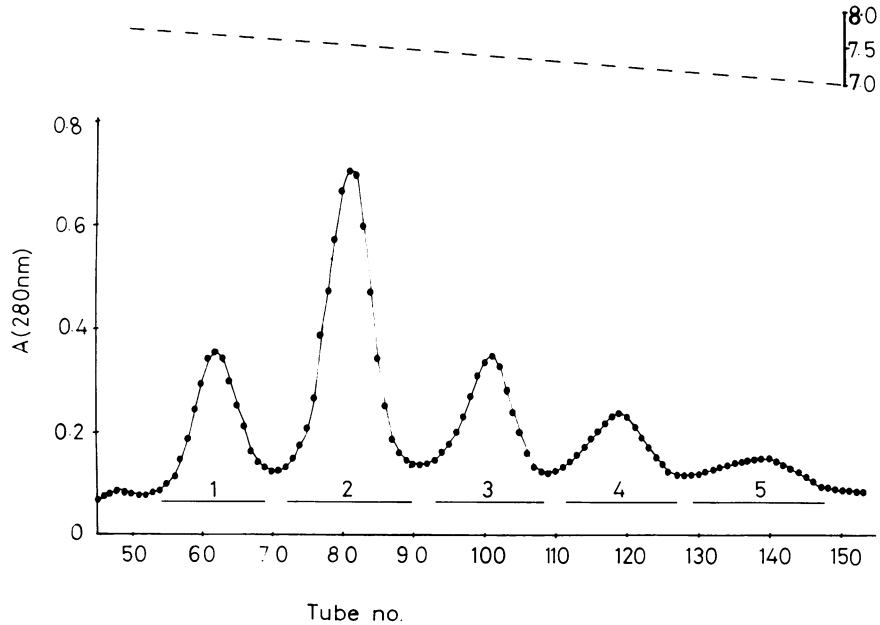

FIG. 2. Elution pattern of newborn's haemolysate on DEAE Sephadex column chromatography, according to Huisman and Dozy (1965). The proportions were:

Fraction 1: Hb A	16·9%
Fraction 2: Hb F	41·0%
Fraction 3: Hb F_3	19·1%
Fraction 4: Hb F variant + some Hb F	14·3%
Fraction 5: Hb F_3 variant	8·6%

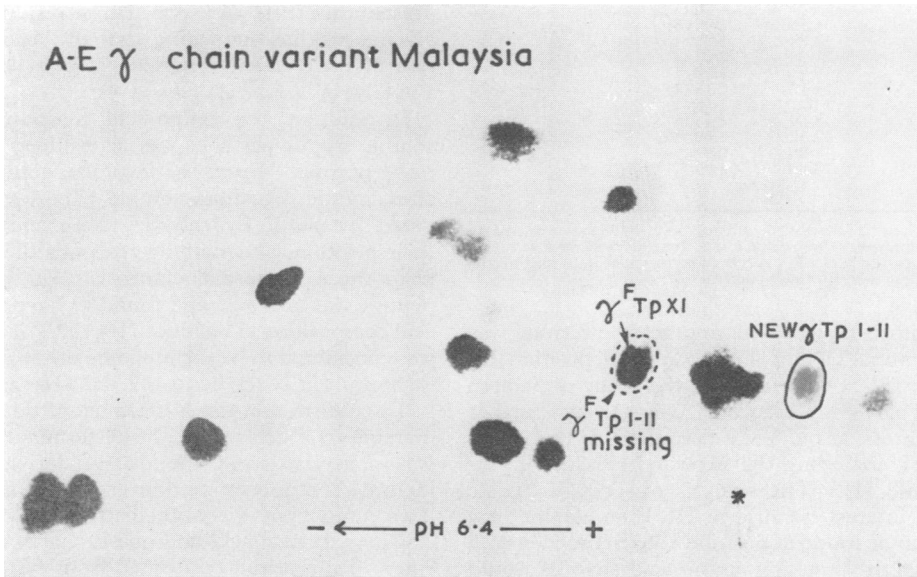

FIG. 3. Peptide chromatogram (fingerprint) of the soluble tryptic peptides of the aminoethylated variant γ-chain from fraction 5 (see Fig. 2). The tryptic peptide γ I–II (residues 1–17) of normal Hb F is missing (see text). A new negatively charged tryptic peptide is noted which corresponds to the tryptic peptide γ I–II of the variant γ-chain.

fingerprint shows a transient yellow on staining with ninhydrin due to the N-terminal glycine residue of γ^FTpI–II. On the present occasion both these features were absent. When the spot was cut out and electrophoresed at pH 3·5, no γTpI–II separated from γTpXI. Furthermore, amino-acid analysis of this spot taken from the original fingerprint showed only the residues of γTpXI. The pH 6·4 fingerprint showed a new peptide, which stained for tryptophan and was more negatively charged than γ^FTpI–II (Fig. 3). Amino-acid analysis showed that all the residues of γ^FTpI–II were present with one exception: there was one glycine residue instead of the expected two (at positions 1 and 16). As the spot had not given a transient yellow colour on treatment with ninhydrin, we assumed that it did not contain the N-terminal glycine. Amino-acid analysis showed an additional peak in the position of cysteic acid or taurine (Table I). Thus we inferred that the amino-acid substitution in the γ-chain was 1 Gly→Cys, and that the cysteine had during handling been converted to cysteic acid and/or taurine.

TABLE I

AMINO-ACID COMPOSITION OF γTpI–II (γ1–17) OF THE Hb F VARIANT (NEGATIVE AT pH 6·4)

Amino Acid	n Moles	Residues	γ^FTpI–II (residues expected)
Cys acid	7·14	0·59 (1)	–
Asp	15·71	1·28 (1)	1
Thr	36·16	2·95 (3)	3
Ser	14·84	1·21 (1)	1
Glu	27·28	2·22 (2)	2
Gly	13·51	1·10 (1)	2
Ala	14·12	1·15 (1)	1
Ile	13·01	1·06 (1)	1
Leu	14·00	1·14 (1)	1
Phe	12·32	1·00 (1)	1
His	11·20	0·91 (1)	1
Lys	23·89	1·95 (2)	2
Trp*	Present		1

* Tryptophan is destroyed during acid hydrolysis preceding amino-acid analysis. Its presence was demonstrated in the fingerprint by fluorescence of the peptide in ultra-violet light and by the positive reaction with Ehrlich's reagent.

To confirm that the missing glycine residue was the N-terminal glycine and not that at position 16, pure γTpII (residues 9–17) was fully separated from γTpXIV (residues 133–144) by further electrophoresis at pH 3·5. It contained all residues of γ^FTpII, including the expected residue of glycine (Table II). The analysis of TpXIV (Table III) is of interest because in Hb F an alanine or a glycine can be found at position 136 (Schroeder *et al*, 1968). In the first case amino-acid analysis would show three alanine residues (136, 138, and 140) and no glycine residues, and the chain would be an $^A\gamma$ chain; in the second there would be two alanine

TABLE II

AMINO-ACID COMPOSITION OF γTpII (γ9–17) OF THE Hb F VARIANT (POSITIVE AT pH 6·4)

Amino Acid	n Moles	Residues	γ^FTpII (residues expected)
Thr	10·86	2·09 (2)	2
Ser	4·62	0·89 (1)	1
Gly	5·12	0·99 (1)	1
Ala	5·02	0·97 (1)	1
Ile	5·20	1·00 (1)	1
Leu	5·58	1·07 (1)	1
Lys	5·18	1·00 (1)	1
Trp*	Present		1

* As in Table I.

TABLE III

AMINO-ACID COMPOSITION OF γTpXIV (γ133–144) OF THE Hb F VARIANT (POSITIVE AT pH 6·4)

Amino Acid	n Moles	Residues	γ^FTpXIV (residues expected)
Thr	9·7	1·05 (1)	1
Ser	27·8	3·03 (3)	3
Gly	10·6	1·15 (1)	1 or 0
Ala	21·6	2·35 (2)	2 or 3
Val	21·5	2·35 (2)	2
Met	2·5	0·27*	1
Leu	9·6	1·04 (1)	1
Arg	8·1	0·88 (1)	1

* Methionine tends to be destroyed on acid hydrolysis preceding amino-acid analysis, and low yields are therefore not unexpected.

residues (138 and 140) and one residue of glycine (136), and the γ-chain would be a $^G\gamma$-chain. In the present case there were two alanine residues and one glycine residue, indicating that the likely description of the γ-chain variant is γ1(NA1) Gly→Cys; 136 Gly, or $^G\gamma$1(NA1) Gly→Cys.

In addition, the amino-acid composition of all soluble tryptic peptides was determined and found to be normal. From the insoluble core remaining after tryptic digestion, soluble peptides were prepared by peptic hydrolysis. With one exception, their positions and staining reactions did not differ from those of normal controls. The exceptional peptide was analysed and found to have the amino-acid composition of residues 110-113. This peptide was considered to be the outcome of unspecific peptic hydrolysis of the bond 109 Val-110 Leu.

To confirm that the N-terminal of this chain was cysteine or a derivative, the N-terminal of 3·1 mg was dansylated and the dansyl derivatives were identified on polyamide thin-layer chromatography. Two components were obtained, one in the position of dansyl cysteic acid and one in that of dansyl taurine. Taurine can be formed from cysteic acid on decarboxylation. It can also be formed from aminoethyl cysteine itself (Shotton and Hattley, 1973), but careful search of paper chromatograms

failed to demonstrate the presence of either of free aminoethyl cysteine or of aminoethyl cysteine attached to γTpI–II.

Analysis of Variant Hb F (Fraction 4). On chain separation of fraction 4 obtained on column chromatography, again both the normal γ-chain and a slightly more negatively charged γ-chain were obtained. The separation was so poor that they were harvested together and analysed. The tryptic fingerprint showed a spot in the position of γTpI–II and γTpXI which stained for tryptophan. No abnormal tryptophan-positive peptide was found. The spot was cut out and re-electrophoresed at pH 3·5 to separate γTpI–II from γTpXI. Two spots separated, one in the position of normal γTpI–II and the other in that of γTpXI. The first gave on amino-acid analysis the residues expected for the normal γ^FTpI–II (Table IV) and the other contained the amino acids of γTpXI plus those of γTpI–II minus one glycine (Table V). It was assumed that as the chain had been aminoethylated, the N-terminal AE-cysteine had been cleaved off the variant γTpI–II on tryptic digestion preceding the fingerprinting of the peptides. To prove that this preparation originally contained N-terminal cysteine, 3·0 mg were dansylated and again dansyl taurine, in addition to a small amount of dansyl glycine was found, which was expected as the preparative (fraction 4) contained the variant γ-chain as well as approximately 18% of the normal γ-chain.

TABLE IV

AMINO-ACID COMPOSITION OF PEPTIDE SEPARATED BY ELECTROPHORESIS AT pH 3·5 FROM γTpI–II OF THE Hb F VARIANT AND γ^FTpXI (γ96–104)

Amino Acid	n Moles	Molar Ratio	Expected for γ^FTpI–II
Asp	8·92	1·17 (1)	1
Thr	19·77	2·61 (3)	3
Ser	9·83	1·29 (1)	1
Glu	17·52	2·30 (2)	2
Gly	14·33	1·89 (2)	2
Ala	9·05	1·19 (1)	1
Ile	7·39	0·97 (1)	1
Leu	8·70	1·14 (1)	1
Phe	8·40	1·10 (1)	1
His	8·18	1·07 (1)	1
Lys	15·80	2·08 (2)	2
Trp*	Present		1

* As in Table I.

The composition is that of γ^FTpI–II (γ1–17).

Discussion

The variant described here is the first haemoglobin with an N-terminal cysteine side-chain. Schroeder *et al* (1973) proposed that there are not only two γ-chain loci, one for the $^G\gamma$ and one for the $^A\gamma$-chain, but that there might be two genes for each of the $^G\gamma$ and for the $^A\gamma$-chains, one responsible for more and one for less of the $^G\gamma$ and $^A\gamma$-chains respectively. These would be $^{Gl}_{m}\gamma$ and $^{G}_{l}\gamma$, and the $^{A}_{m}\gamma$ and $^{A}_{l}\gamma$-chain genes respectively; the present gene producing 18·8% of the total γ-chains would then be a $^{G}_{m}\gamma$-chain gene.

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TABLE V

MIXTURE OF PEPTIDES FROM WHICH, BY ELECTROPHORESIS AT pH 3·5, γ^FTpI–II (γ1–17) HAS BEEN SEPARATED

Amino Acid	Total n Moles	n Moles for γ^FTpI–II	Molar Ratio	Residues for γ^FTpI–II	n Moles for γTp^{F}XI	Molar Ratio	Residues for γ^FTpXI
Asp	61·69	18·99	1·00 (1)	1	42·70	1·84 (2)	2
Thr	52·18	52·18	2·75 (3)	3	—	—	—
Ser	21·33	21·33	1·12 (1)	1	—	—	—
Glu	67·00	38·01	2·0 (2)	2	28·99	1·25 (1)	1
Pro	19·70	—	—	—	19·70	0·85 (1)	1
Gly	20·59	20·59	1·08 (1)	2	—	—	—
Ala	24·75	24·75	1·30 (1)	1	—	—	—
Val	24·07	—	—	—	24·07	1·03 (1)	1
Ile	16·64	16·64	0·88 (1)	1	—	—	—
Leu	28·00	13·84	0·75 (1)	1	14·16	0·61 (1)	1
Phe	39·21	18·99	1·00 (1)	1	20·22	0·87 (1)	1
His	36·12	12·93	0·68 (1)	1	23·19	1·00 (1)	1
Lys	64·49	38·01	2·0 (2)	2	26·48	1·14 (1)	1
Trp*		Present		1			

* As in Table I.

The composition is γ^FTpXI (γ96–104) plus γ^FTpI–II minus one Gly. It is suggested in the text that this arises from the splitting off of Aminoethylated Cys from γTpI–II of the Hb F variant during tryptic digestion.

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