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A new cause of haemolytic anaemia in the newborn

A description of an unstable fetal haemoglobin: F Poole, $\alpha_2{}^G\gamma_2$ 130 tryptophan → glycine

J. P. LEE-POTTER, R. A. DEACON-SMITH, M. J. SIMPKISS, H. KAMUZORA, AND H. LEHMANN

From the Departments of Haematology and Paediatrics, Poole General Hospital, Poole, Dorset, and the MRC Abnormal Haemoglobin Unit, Department of Clinical Biochemistry, Addenbrooke's Hospital, Hills Road, Cambridge

SYNOPSIS In a newborn twin with haemolytic anaemia an unstable fetal haemoglobin was found to be the cause. The anaemia improved spontaneously with the disappearance of the fetal haemoglobin. The new Hb F ($\alpha_2\gamma_2$) variant was shown to have a glycine at position 130 of the 146 residues of the γ chain. This position is inside the globin molecule and in all known normal globins it is occupied by a residue with a bulky hydrophobic side chain. Its replacement by glycine which has no side chain would be expected to cause instability. The human γ-chains may either have a glycine or an alanine at position 136. Evidence is brought forward to suggest that in the abnormal chain position 136 is occupied by glycine.

During the last decade nearly 50 haemoglobin variants have been reported in which the structural abnormality leads to instability of the molecule (Lehmann, 1974). Such variants can involve the α or the β chain of which haemoglobin A is composed. The present report will describe the characterization of the first unstable haemoglobin in which the molecular abnormality is located in a γ chain. The new variant is named Hb F Poole.

The Finding of an Unstable Haemoglobin Haemolytic Anaemia in a Newborn

D.B. was the second born of premature dissimilar male twins born in February 1974. He was delivered by breech delivery following 36 weeks of an uneventful gestation. Both twins weighed 5 lb each. D.B. was slightly irritable for the first few days and became jaundiced to a serum bilirubin level of 15·9 mg/100 ml between the third and seventh days. At 11 days his urine was noticed to be dark. Red cells were not found microscopically, but one specimen contained small amounts of haemoglobin detectable on spectrophotometry. Vitamin K had not been administered. The infant was 0 Rh (D) negative, and the mother 0 Rh (D) positive. She had no antibodies during pregnancy, and the infant's Coombs test was negative.

The haemoglobin concentration at this time was 13·1 g/100 ml and occasional contracted cells were seen on the blood film. The G-6-PD content of the erythrocytes and prothrombin was normal. On electrophoresis the haemoglobin did not contain any detectable abnormal fractions. Tests for unstable haemoglobin (heat, Dacie, Grimes, Meisler, Steingold, Hemsted, Beaven, and White, 1964; isopropanol, Carrell and Kay, 1962) indicated the presence of an unstable haemoglobin. The ascorbate cyanide test (Jacob and Jandl, 1966) was positive initially and remained so until the unstable haemoglobin could no longer be detected by the isopropanol test. This test was designed to detect deficiencies in the hexose monophosphate shunt but may also be positive when an unstable haemoglobin is present. An abnormal number of erythrocytes were found to contain Heinz bodies on incubation with acetylphenylhydrazine. Schumm's test showed the presence of methaemalbumin. Nevertheless, the infant gained weight and remained clinically well. On discharge at the age of 4 weeks his Hb level was 10·4 g/100 ml. When re-examined at 6 weeks his weight was 8 lb 10 oz.

His twin brother became jaundiced after birth. Tests for an unstable haemoglobin gave normal

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results. There was no reticulocytosis and the jaundice rapidly resolved after it had reached a bilirubin level of 13·2 mg/100 ml at five days. There was no clinical doubt that the jaundice was other than physiological. Otherwise the propositus parents', his half sister's, and his twin brother's blood revealed no abnormalities. The mother had a parent of negroid ancestry but the child's other grandparents are Caucasian. The parents were not married and it was not possible to contact the family again when it was intended to re-investigate the twins.

Identification of the Unstable Haemoglobin

The haemoglobin was incubated at 37°C with isopropanol (Carrell and Kay, 1972) for five minutes to concentrate differentially the unstable haemoglobin fraction in the precipitate. The precipitate was dissolved in 0·1 N HCl in 8 M urea and the haem removed by acid precipitation at −20°C using 1·5% v/v HCl-acetone. The precipitate was washed twice with acid acetone and then three times with acetone, all at −20°C (Rossi-Fanneli, Antonini, and Caputo, 1958). The precipitated globin (24·7 mg) was digested for two hours in 2·5 ml 0·1 M $(NH_4)HCO_3$ pH 8·5 in the presence of 0·5 mg trypsin (TPCK treated).

After two hours the pH was lowered to 6·5 with 0·5 M acetic acid to precipitate the insoluble core which was removed by centrifugation. The supernatant containing the soluble tryptic peptides was dried in a dessicator containing P_2O_5, and applied to Whatman 3 MM papers and fingerprints were prepared as previously described (Sick, Beale, Irvine, Lehmann, Goodall, and MacDougall, 1967) except that for some of the preparative fingerprints chromatography was carried out for 48 hours instead of the usual 18 hours. Diagnostic fingerprints were stained with 0·2% ninhydrin in acetone as well as with reagents specific for histidine (Pauly), tyrosine (1-nitroso-2-naphthol), sulphur containing amino acid (platinic iodine), tryptophan (Ehrlich), and arginine (Sakaguchi) in a sequence outlined by Easley (1965).

Preparative fingerprints were stained with 0·02% ninhydrin in acetone. All peptides from the preparative fingerprints were purified either at pH 3·5 or pH 9·0 or both before elution and amino acid analysis, unless stated otherwise. All peptides were eluted with fresh distilled constant boiling 6N HCl into capillary tubes which had been cleaned by soaking them in chromic acid overnight before rinsing them with distilled water and drying them at 140°C for two hours. The tubes were sealed and hydrolysis was carried out at 108°C for 24 hours then they dried down and loaded on a Locarte amino acid analyzer. The supernatant remaining after incubation with isopropanol was treated with acid acetone and the precipitated globin reacted with cyanogen bromide (Gross and Witkop, 1962). The γCB-3 (γ134-146) fragment was isolated by electrophoresis and paper chromatography and its amino acid composition determined. A comparison of the molar ratios of glycine/alanine in the tryptic peptide (Tp) XIV (γ133-144) from the isopropanol precipitate and the γCB-3 fragment from the supernatant provided information to suggest whether residue 136 in the abnormal fetal haemoglobin was alanine or glycine.

Results

On ninhydrin staining the fingerprint pattern of the precipitated haemoglobin was that of normal Hb F (see fig). Staining reactions for specific amino

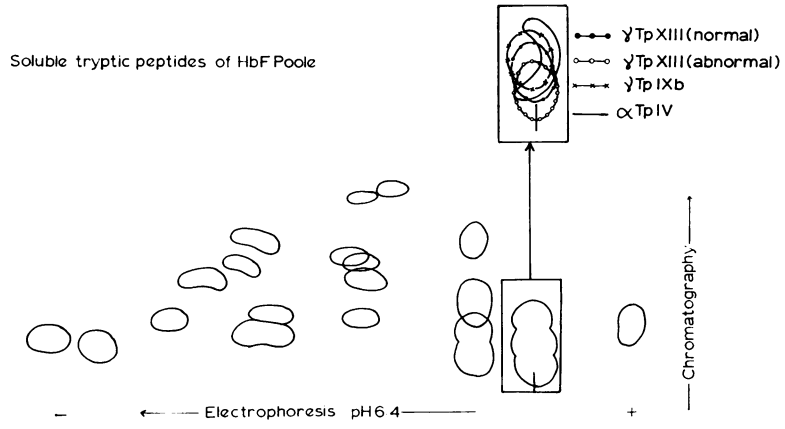

Fig *Fingerprint of the soluble tryptic peptides of Hb F Poole obtained by precipitation with isopropanol*
Note that normal Hb F is partially precipitated in the process

	γTp XIII (Abnormal)		Expected from Normal γTp XIII	γTp IXb	Expected from Normal γTp IXb
	n moles	*Molar Ratio*			
Asp	17·9	—	—	2·1	2
Thr	9·9	0·9	1	—	—
Ser	12·3	1·1	1	—	—
Glu	38·3	3·5	4	—	—
Pro	8·6	0·8	1	—	—
Gly	12·9	1·2	0	—	—
Ala	13·8	1·3	1	—	—
Val	12·9	1·2	1	—	—
Leu	15·0	—	—	1·8	2
Phe	10·2	0·9	1	—	—
His	4·7	—	—	0·6	1
Lys	16·3	(1·0)	1	(0·7)	1
Trp	Negative	—	1	—	—
	One residue = 10·8 n moles			One residue = 8·2 n moles	

Table I *Amino acid composition of the mixture of the patient's tryptic peptides γIXb (residues 77-82) and the abnormal γXIII (residues 121-132)*

acids were also identical to those of normal controls, except that on staining for tryptophan, the intensity of the γTp XIII (residues 121-132) was diminished. It was therefore necessary to analyse all soluble tryptic peptides. Their amino acid composition gave molar ratios expected for normal α and γ chains, except that in the γTp XIII region, the amino acid composition without further purification yielded an extra residue of glycine, plus the molar ratios corresponding to a mixture of normal γTp XIII (residues 121-132), γTp IXb (residues 77-82), and αTp IV (residues 17-31). To determine where the extra residue of glycine came from the area around γTp XIII was cut out from a preparative fingerprint which had been kept in chromatography tanks for 48 hours and re-electrophoresed at pH 9·0. At this pH, αTp IV separated from γTp XIII and γTp IXb. The amino acid composition of these two spots indicated that glycine was located in the γTp XIII/γTp IXb mixture and not in αTp IV. Table I shows the amino acid composition of the mixture of the abnormal γTp XIII and γTp IXb. It will be seen that no tryptophan is present and there is an additional glycine in γTp XIII (residues 121-132). Although tryptophan can be destroyed during acid hydrolysis preceding amino acid analysis, its presence can usually be inferred from a positive staining reaction on the fingerprint. This was absent here, confirming a substitution of tryptophan by glycine. Except for C terminal lysine γTp XIII (residues 121-132) and γTp IXb (residues 77-82) have no residues in common. Because of shortage of material and further purification caused losses, it was decided to analyse the two peptides together. To investigate the abnormal γTp XIII by itself, another preparative fingerprint was prepared and the abnormal γTp XIII region treated as above. It was further re-electrophoresed at pH 3·5 in an attempt to separate it from γTp IXb but the material was lost in the process and the yield was too small for analysis. Table II shows the amino acid composition of a mixture of normal γTp XIII and the abnormal γTp XIII from a fingerprint which had been kept in chromatography tanks for 24 hours. The γTp XIII was purified by electrophoresis at pH 9·0 and pH 3·5 to remove αTp IV and γTp IXb respectively. The normal and the abnormal γTp XIII peptides had not separated but the presence of the additional glycine and the diminished intensity of Ehrlich's reaction for tryptophan further supports the supposition that the amino acid substitute is γ130 (H8) tryptophan → glycine. In view of the fact that man possesse two γ chains, one with alanine and the other with glycine at position γ136 (Schroeder, Huisman, Shelton, Shelton, Kleihauer, Dozy, and Robertson, 1968), it had to be determined whether this position was occupied by alanine or glycine in Hb F Poole, ie, whether the Hb F Poole γ chain was a $^{G}\gamma$ or an $^{A}\gamma$ chain. Unfortunately, the precipitation procedure for unstable haemoglobin

	γTp XIII (normal + abnormal)		Expected from Normal γTp XIII
	nmoles	*Molar Ratio*	
Thr	10·7	1·0	1
Ser	11·1	1·1	1
Glu	37·8	3·6	4
Pro	11·8	1·1	1
Gly	5·4	0·5	0
Ala	12·2	1·2	1
Val	10·9	1·0	1
Phe	9·2	0·9	1
Lys	10·7	1·0	1

Table II *Amino acid composition of the mixture of the patient's normal and abnormal tryptic peptides γXIII (residues 121-132)*

One residue = 10·4 nmoles

(heat and isopropanol), though not precipitating Hb A, partially precipitate normal Hb F. Thus, it was not possible to separate completely Hb F and Hb F Poole. But Hb F Poole was largely precipitated and the normal Hb F only partially by isopropanol. In the fragments γ134-146 and γ133-144 of the isolated $^{G}\gamma$ chain there are two alanines and one glycine (136 Gly, 138 and 140 Ala) and the glycine/alanine ratio is 0·50; and in the isolated $^{A}\gamma$ chain there are three alanine residues in the fragments and no glycine, so that the glycine/alanine ratio is 0. A lower glycine value was obtained from the supernatant in which some abnormal γ chain was present as an impurity than in the precipitate. In the supernatants the values were Gly 0·76, Ala 2·36 and in the preipitate Gly 0·96, Ala 2·17, the glycine alanine ratio being in the supernatant 0·32 and 0·44 in the precipitate. From these differences it was concluded that the abnormal chain was $^{G}\gamma$, ie, it contained glycine at position γ136 rather than alanine. As the glycine/alanine ratio is high in the newborn in any case, an increase in the proportion of glycine in the precipitate yields less impressive figures for a $^{G}\gamma$ chain than what it would have been for an $^{A}\gamma$ chain.

Discussion

Hb F Poole represents the first observation of an unstable fetal haemoglobin. It is also the first variant to affect residue γ130 (H8). Position H8 of the polypeptide chains of human haemoglobins and myoglobin is occupied by an amino acid with a bulky hydrophobic side-chain, Leu in α, Tyr in β, Trp in γ, and Met in Mb (Dayhoff *et al*, 1972). The only other variant that is known to involve residue H8 is haemoglobin Wien ($\alpha_2\beta_2^{130Asp}$) (Lorkin, Pietschmann, Braunsteiner, and Lehmann, 1974), and like Hb F Poole, is unstable and associated with haemolytic anaemia. Glycine has no side chain.

Since the amount of Hb F in the circulation normally decreases to less than 2% of the total haemoglobin over the first six months, the molecular abnormality should have disappeared by this time. Thus, Hb F Poole was only associated with a haemolytic process during the first few months of extrauterine life. Due to the transient appearance of Hb F Poole and the difficulties in obtaining large volumes of blood from neonates, more extensive data in sequence could not be obtained.

This is the first example of an unstable fetal haemoglobin haemolytic anaemia. It may well be that some unexplained haemolytic anaemias in newborn infants might be caused by an unstable fetal haemoglobin, and that testing the blood for this possibility would materially assist diagnosis and prognosis. Hb F Poole was heavily precipitated in less than five minutes in the isopropanol test, where normal Hb F forms only a cloudy precipitate within five minutes and Hb A none in less than 10 minutes.

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Appendix

Haematological Progress of the Patient

Age (weeks)	*Hb (g/100 ml)*	*MCV (fl)*	*MCH (pg)*	*Reticulocytes (%)*	*WBC × 10³/μl*
2	13·1	103	37·4	5·0	18·7
3	10·2	95	35·9	5·0	16·3
4	10·4	98	37·6	—	10·7
6	10·5	91	35·6	7·0	11·1
16	12·4	72	24·5	1·4	9·6