Hereditary Non-spherocytic Haemolytic Anaemia with Post-splenectomy Inclusion Bodies and Pigmenturia Caused by an Unstable Haemoglobin Santa Ana-β88 (F4) Leucine→Proline

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There have now been described a number of congenital non-spherocytic haemolytic anaemias, several of which are caused by one of a number of unstable haemoglobins. In one group the instability of the haemoglobin molecule arises from a loss of hydrophobic contact which helps to bind the haem to the globin by Van der Waals forces. One example in haemoglobin Köln is the replacement of β98 valine by methionine (Carrell, Lehmann, and Hutchison, 1966). The 98th residue of the 146 of the β chain is the fifth of a chain of six amino acids (β94–99) which link the helices F (β85–93) and G (β100–117) of the β chain, and its helical notation is therefore FG5; it is in direct hydrophobic contact with the haem group (Perutz *et al.*, 1968). Another example is haemoglobin Sydney (Carrell *et al.*, 1967). Here a side chain of valine at β67 (E11), which is another direct haem contact, is replaced by the shorter side chain of alanine. Such an apparently small change in the Van der Waals forces which keep the haem in its globin pocket suffices to loosen the haem-globin combination and to make the haemoglobin unstable.

Another type of instability is caused by the insertion of the imino acid proline in the middle of a helically arranged sequence of amino acids in the haemoglobin molecule. The resulting distortion of the tertiary structure of the molecule interferes with its stability, and two such unstable haemoglobins are haemoglobin Genova where the B helix is interrupted by a substitution of β28 (B10) leucine by proline (Sansone, Carrell, and Lehmann, 1967), and haemoglobin Bibba where the same substitution in position α 136 (H19) has been demonstrated by Kleihauer *et al.* (1968).

Present Investigations

We want to describe an unstable haemoglobin which is a third example for both types of unstable haemoglobin. It shows a neutral mutation at a haem contact resulting in a loss of hydrophobic forces binding the globin to the haem, and this mutation is a substitution of leucine by proline in a helical region of the haemoglobin molecule.

Case Reports

Case 1. The propositus was born in 1926. Anaemia was noted when he was in hospital for supposed typhoid fever in 1927. Splenomegaly and anaemia were noted at that time. Hb '40%', RBC 2·7 M/cu. mm. Widal test positive 1:40. Laboratory reported marked anisocytosis and slight polychromasia. Urine analysis was said to reveal an amber urine. However, the patient's mother stated that the urine became black around that time, or was noticed to be black around that time. He was in hospital again in 1929. Jaundice and splenomegaly were noted, and the urine was reported to be dark brown. RBC were 3·9 M/cu. mm. Splenomegaly and jaundice were variable after that but became persistent after the age of 12. He was rejected by the U.S. Air Force for enlistment in 1943 because of jaundice and splenomegaly. The spleen progressively increased in size and in 1947 was at the iliac crest. Hb at that time was 9·0 g./100 ml.; RBC 3·5 M/cu. mm. Marked anisocytosis and basophilic stippling were noted. Icterus index 22, osmotic fragility normal, and platelets were reduced—15,000/cu. mm. on one count, and 20,000 on another. Urine analysis revealed dark brown urine. In 1948 at the age of 22 years, splenectomy was performed. The spleen weighed 1920 g. Six months after splenectomy, Hb was 12 g./100 ml. and the RBC 4·4 M/cu. mm. In 1953 Hb was 13·4/100 ml., RBC 3·27 M, reticulocytes 5·6%, haematocrit 49·5%, Coombs test negative, and serum bilirubin 2·4 mg./100 ml. He was first seen by one of us (R.W.O.) in 1959. He was found to have mild icterus and a splenectomy scar but no other abnormalities. Hb 14 g./100 ml. and haematocrit 44%.

The urine was coffee-coloured. On the Wright's stain of the peripheral blood smear there was marked anisocytosis, polychromasia, slight macrocytosis, numerous Howell-Jolly bodies, occasional Pappenheimer bodies, and basophilic stippling. Reticulocytes were 10% but almost every red cell had a variable-sized inclusion, many of them large and centrally located. In October 1960, Hb fell to 9·5 g./100 ml. during an episode of acute pneumonia. He recovered from this spontaneously. The haematological values returned to his usual levels spontaneously, and Hb has remained in the 12 to 14 g./100 ml. range since that time. He is otherwise in excellent health.

Case 2. A daughter of the propositus was born in 1952. Her mother reports that dark urine was noted within several months after birth. Anaemia, jaundice, and splenomegaly became evident at about 18 months of age. She was first seen in 1959 at which time the spleen was enlarged to five fingers below the costal margin. Haematocrit was 33%. The peripheral blood smear revealed marked anisocytosis, polychromasia, and basophilic stippling, but there were no Howell-Jolly bodies or Pappenheimer bodies. Reticulocytes numbered 20%, and no inclusion or Heinz bodies were seen on thorough review of new methylene blue, crystal violet, or Nile blue sulphate stains. In October 1962, her spleen, weighing 552 g., was removed. Biopsy of the liver revealed golden pigment in the hepatic cells. There were negative stains for homogentisic acid, negative buffalo black stain for Hb, negative PAS stain for lipochromes, negative Schmorl's stain for adrenochromes, and negative tests for bilirubin. Intra-erythrocytic inclusion bodies were first seen on the fourth day, increasing to 18% on the eighth day and 58% three months later, at which time Hb was 12·2 g./100 ml., haematocrit 37%, and serum bilirubin 0·6 mg./100 ml. Haematological values have remained stable since that time, and she has shown normal growth and development.

Case 3. A son of the propositus was born in 1957. Urine was noted to be black within two months of birth, and jaundice and anaemia became evident at 18 months of age When first seen in 1959, the spleen was three fingers below the costal margin. Hb was 8·1 g./100 ml., haematocrit 30%, reticulocytes 25%. The red cell morphology was identical to his sister's (Case 2), and no intra-erythrocytic inclusions were seen. In 1965, a spleen weighing 414 g. was removed. Intra-erythrocytic inclusions were noted on the fourth day and increased gradually thereafter. The haematocrit rose to 38%, reticulocyte count fell to 3%, and approximately 80% of the red cells contained intra-erythrocytic inclusions.

Case 4. A daughter of the propositus. She was thoroughly examined. She had no jaundice or splenomegaly. Haematocrit 38%. Red cell morphology was normal. Reticulocyte stain revealed no intra-erythrocytic inclusions.

Case 5. A son of the propositus was born in December 1967. Peripheral blood smear suggests that this child is affected.

Investigations of the other relatives were as follows:

Father of the propositus: haematocrit 48%; no enlargement of the spleen, no jaundice, red cell morphology normal; reticulocyte stain revealed no abnormalities.

Mother of the propositus: haematocrit 46%; no jaundice; no splenomegaly; no abnormalities on Wright's stain or supravital stain of peripheral blood smear.

Brother of the propositus: haematocrit 50%; no jaundice, no splenomegaly.

Wife of the propositus and mother of Cases 2, 3, 4, and 5: no jaundice, no splenomegaly; haematocrit 40%; peripheral blood smear was not remarkable.

Investigation of the Haemoglobin

On incubating the blood of the propositus at 50° C. for one hour according to Dacie *et al.* (1964) a copius precipitate was formed indicating that the cause of his Heinz body anaemia was an unstable haemoglobin. When the haemolysate was examined by paper electrophoresis at *p*H 9·9, an additional band to those of Hb A and Hb A$_2$ was seen moving behind the Hb A and faster than Hb A$_2$ (Fig. 1). On starch gel electrophoresis, according to Poulik (1957), a second additional band was noted in the position of free α chains as is often noted when β chain unstable haemoglobins are examined by that method (Huehns and Shooter, 1965). The haemoglobin was isolated by repeated paper electrophoresis using Tris buffer *p*H 8·9 (Cradock-Watson, Fenton, and Lehmann, 1959) until it was free from Hb A and Hb A$_2$. It was then noted that there was a discrepancy between the determination of the cyan-methaemoglobin and of the protein concentration by refractometry. When untreated haemolysates were adjusted to a 5% concentration of cyanmethaemoglobin, the protein concentration was 5·6%. After separation and purification by electrophoresis of the Hb A and of the abnormal haemoglobin the protein content of the 5% (cyan-methaemoglobin) solution Hb A was 5·1% but that of the abnormal fraction was 9·8%, indicating that it had only two haem groups per molecule of haemoglobin. A similar gross impairment of haem-binding has been reported for Hb Gun Hill in which five amino acid residues are deleted in the β chain (Bradley, Wohl, and Rieder, 1967), and impairment of haem binding is considered a general feature of unstable haemoglobins (Carrell *et al.*, 1967), the loss of haem contributing to precipitation of Heinz bodies (Jacob *et al.*, 1968).

For the examination of the amino acid sequence of the abnormal haemoglobin the purified solution was incubated at 50° C. A whitish precipitate indicating a low haem content appeared and was then washed repeatedly with *p*H 7 phosphate buffer
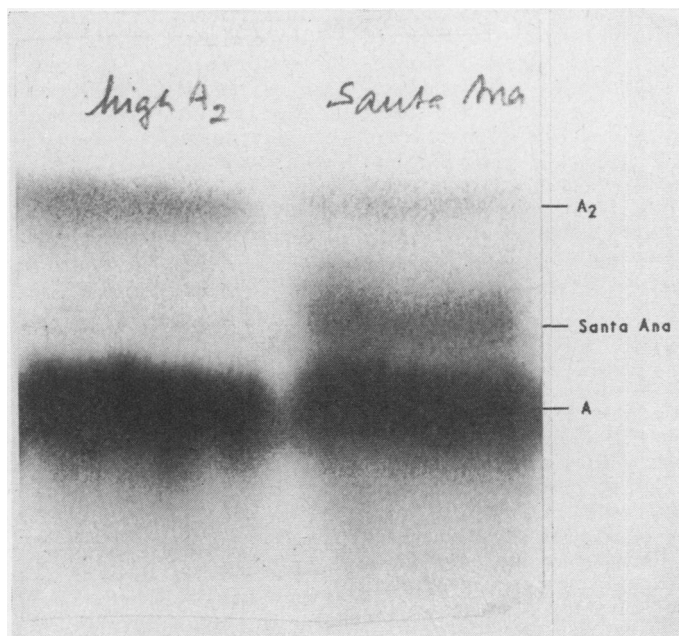
FIG. 1.   Paper electrophoresis (TRIS buffer, pH 8·9) of the haemoglobin solution of the propositus with Hb A and Hb A_2 as control.
Hb Santa Ana is seen as an additional band in the case of the propositus.

of 50° C. temperature. The globin was amino-ethylated (Jones, 1964) and then purified by gel filtration on Sephadex G25 and isolated by freeze-drying. Part of the haemoglobin was separated into its α and β chains according to Clegg, Naughton, and Weatherall (1966). For this the precipitated unstable haemoglobin was dissolved at a concentration of about 5% w/v, in a solution containing 1% pyridine, 0·05M β-mercaptoethanol in 8M urea, pH 8·8, and incubated at room temperature for 4 hours. This treatment was intended to reduce any intermolecular disulphide bonds which might have formed during the heat treatment of the haemoglobin. Globin was then precipitated by adding the solution dropwise to 1·5% v/v concentrated hydrochloric acid in acetone at $-20°$ C., washed with acetone at $-20°$ C., and dried under nitrogen. When chromatography on a column of carboxymethyl cellulose in 8M urea was then carried out according to Clegg et al. (1966), much of the protein failed to bind to the column, nevertheless approximately 50% of the theoretical yields were obtained in some separations.

The aminoethylated (AE) globin and the AE α and β chains were digested with trypsin, and 'fingerprints' (peptide chromatograms) were prepared which were stained with ninhydrin and specific reagents for histidine, arginine, tyrosine, tryptophan, and sulphur-containing amino acids (methionine and β-aminoethylcysteine). The methods used have recently been summarized by Sick et al. (1967) and by Beale (1967).

Part of the globin molecule, the so-called core comprising residues 93–140 of the α chain and 83–120 of the β chain, remains insoluble or only partly soluble after tryptic digestion. Aminoethylation converts the cysteines at position 104 of the α chain and positions 93 and 112 of the β chain into β-aminoethylcysteine, which as a basic amino acid is then acted upon by trypsin. On tryptic digestion of the AE globin, part of the α chain core, and the whole of the β chain core, are rendered soluble. Six extra peptides are found on the fingerprint of the AE globin, αTpXI (α93–99); αTpXIIa (α100–104); βTpX (β83–95); βTpXI (β96–104); βTpXIIa (β105–112), βTpXIIb (β113–120), and traces of βTpXa (β83–93).

In the fingerprint of AE globin of the unstable haemoglobin all the peptide spots gave the expected staining reactions and were in their usual positions except for the spot representing βTpX, which had a normal electrophoretic mobility, but a lower chromatographic mobility than usual. When the electrophoretic separation is carried out at pH 3·5, βTpX has the same electrophoretic mobility as βTpI (β1–8). In such fingerprints of AE globin
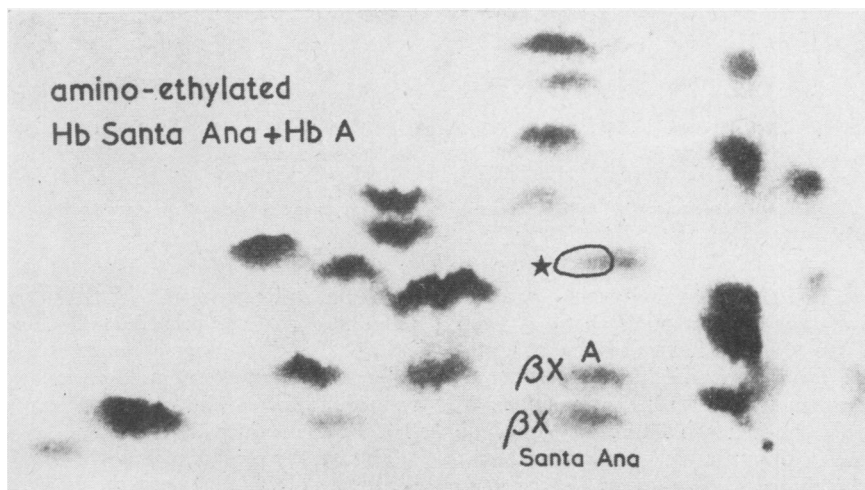
FIG. 2. Comparison of Hb A and Hb Santa Ana. Fingerprint of a mixture of AE globin A and AE globin Santa Ana—pH 6·4. Two βTpX spots can be seen, of which the upper corresponds to β^ATpX, and the lower to βTpX of Santa Ana. The spot marked with an asterisk represents β^ATpXa (residues β83–93).

from Hb A, βTpX is located above βTpI. The abnormal βTpX was however located just below βTpI.

Table I lists the chromatographic mobility of βTpX relative to βTpIV, for the unstable haemoglobin, Hb A, and two abnormal haemoglobins which do not have amino acid substitutions in this peptide. Fig. 2 shows the fingerprint of a mixture of AE Hb A and of the AE unstable haemoglobin. Two βTpX spots, both staining for histidine and β-aminoethylcysteine, are present; the upper corresponding to β^ATpX and the lower to the abnormal βTpX. This clearly shows that the unstable haemoglobin differs from Hb A in residues 83–95 of the β chain. The fingerprint of the tryptic digest of the AE α chain did not show any abnormalities.

Table II shows the amino acid composition of the abnormal βTpX isolated from preparative fingerprints of the AE globin and AE β chain. In the first case the peptide was contaminated with αTpI–II (residues 1–11 of the α chain) and the figures are corrected for contamination. The

second set of figures is the amino acid composition of the pure peptide from the isolated AE β chain. In the abnormal peptide there was only one residue of leucine instead of two and one residue of proline, which is normally absent from β^ATpX. The unstable haemoglobin therefore differs from HbA by a substitution of proline for leucine in βTpX.

There was also an abnormality in the amounts of threonine and glutamic acid found. Normally they should be in the ratio of 2:1, but approximately equal amounts were found. This may indicate a partial substitution of threonine by glutamine, which would be converted into glutamic acid on

TABLE I
CHROMATOGRAPHIC MOBILITIES OF βTpX RELATIVE TO βTpIV

Haemoglobin	Santa Ana	A	K-Ibadan	Köln
	0·073	0·25	0·31	0·22
	0·073	0·31	0·22	0·26
	0·047	0·25	0·28	0·22
	0·115	0·24	0·24	0·21
Average	0·077	0·26	0·26	0·23

TABLE II
AMINO ACID COMPOSITION OF ABNORMAL βTpX

Amino Acid	μ Moles		No. of Residues		
	AE Globin*	AE β Chain	AE Globin	AE β Chain	AE β^ATpX
Asp	0·033	0·024	1·0	1·0	1
Thr	0·052	0·038	1·6	1·6	2
Ser	0·034	0·023	1·1	1·0	1
Glu	0·049	0·038	1·5	1·6	1
Pro	0·033	0·018	1·0	0·7	0
Gly	0·041	0·025	1·3	1·0	1
Ala	0·031	0·033	1·0	1·3	1
Leu	0·034	0·024	1·1	1·0	2
Phe	0·026	0·018	0·8	0·7	1
1 residue	0·032	0·024			

* Corrected for contamination with αTpI–II.

Note: AE β^ATpX contains 3 basic amino acid residues: Lys, His, AE Cys. The presence of histidine and aminoethylcysteine in the abnormal βTpX was indicated by positive staining reactions, and as the peptide was a tryptic peptide (not staining for arginine) its carboxy terminal amino acid was lysine.

	Ac 1	↓		Ac 2			↓	Ac 3			↓	Ac 4	↓	Ac 5
Residue	Gly	Thr	Phe	Ala	Thr	LEU	Ser	Glu	Leu	His	AE Cys	Asp		Lys
Sequential number in β chain	83	84	85	86	87	88	89	90	91	92	93	94		95
Helical notation	EF7	EF8	FI	F2	F3	F4	F5	F6	F7	F8	F9	FGI		FG2

FIG. 3. The amino acid sequence of β^ATpX. Arrows indicate cleavage on partial acid hydrolysis into fragments Ac 1–5. The leucine at position β88 (F4) is replaced by proline in Hb Santa Ana.

acid hydrolysis, in addition to the leucine→proline mutation. Both, the γ chain of Hb F ($\alpha_2\gamma_2$), and the δ chain of Hb A_2 ($\alpha_2\delta_2$) have a residue of glutamine instead of one of threonine at position 87.

There are two leucines in β^ATpX at positions 88 and 91 (Fig. 3), and in order to determine which one was substituted in the unstable haemoglobin, both β^ATpX and the abnormal βTpX were subjected to partial hydrolysis in 0·25N acetic at 110° C. for 12 hours (Ingram and Stretton, 1962). The hydrolysates were fractionated by preparative fingerprinting at pH 6·4. The various fragments were eluted from ninhydrin stained fingerprints, hydrolysed, and analysed for their amino acid content by paper electrophoresis at pH 2 (formic acid, acetic acid, water 1:4:45 by volume) or by the amino acid analyser. Paper electrophoretograms were stained with ninhydrin-collidine. It can be seen in Fig. 3 that five fragments are expected from partial acid hydrolysis of βTpX (Ac 1–5). Ac 2 (β84–89) was not found in either case, possibly because it failed to react sufficiently with ninhydrin to be detectable. Ac 3 (β90–93) was found to contain leucine in both cases. As the leucine at position 91 was present in the abnormal βTpX, it followed that the other leucine at position 88 was the site of the substitution.

The amino acid composition of the other tryptic peptides of the β chain of the abnormal haemoglobin was determined, except that of βTpIV (β31–40) of which not enough material was obtained. No evidence for any further substitutions was found.

The haemoglobin under investigation is therefore a new unstable variant which we have called Hb Santa Ana—β88 (F4) Leucine→Proline.

Discussion

The family study suggests that the newly recorded Hb Santa Ana has arisen in the propositus as a new mutation. Similar observations were made in the case of Hb Hammersmith (β42 (CD1) phenylalanine→serine), when in the only two cases known the variant was not found in either parent (Dacie et al., 1967), and a child with Hb M Hyde Park (β92 (F8) histidine→tyrosine) described by Becroft et al. (1968); and we also know of at least one case of

Hb Köln and one of Hb M Saskatoon (β63 (E7) histidine→tyrosine), respectively, where the variant was not found in either parent. It is possible that before the amenities of modern treatment became available, affected children were less likely to survive and to produce offspring.

Though the proline residue β88 in Hb Santa Ana occurs in a helical portion of the molecule, F4, it may not necessarily cause a change in the conformation of the main chain (Perutz and Lehmann, 1968), and it may be more important that Hb Santa Ana has lost at position βF4 a leucine side chain which is in contact with the haem group, a leucine that is found in this position in all known mammalian haemoglobins. Such a loss of hydrophobic contact can be expected to loosen the hold of the globin on the haem and permit water to enter the haem pocket causing formation of methaemoglobin and denaturation of the globin resulting in intracellular precipitation (Carrell et al., 1967; Jacob et al., 1968).

Though the mutation in Hb Santa Ana does not add an additional positive charge to the amino acid sequence, the variant shows an increased positive charge on electrophoresis at alkaline pH. This was also noted in other unstable haemoglobins with neutral mutations, viz. Hb Köln and Hb Sydney, though it is noteworthy that in Hb Genova the leucine→proline mutation does not cause it to differ from Hb A on electrophoresis. It is obvious that secondary changes in the conformation of the molecule must be the cause of the charge changes seen in Hb Köln, Hb Sydney, and Hb Santa Ana.

Summary

A congenital non-spherocytic haemolytic inclusion (Heinz) body anaemia was found in a man, and some of his children, but not in his parents. The cause was found to be an unstable haemoglobin with impaired haem binding. This haemoglobin showed a replacement of leucine by proline in the 88th position of the β chain—the fourth residue of the F helix. The haemoglobin has been named Santa Ana.

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