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Abnormal Haemoglobins in Iran. Observation of a New Variant— Haemoglobin J Iran ($\alpha_2\beta_2$ 77 His $\rightarrow$ Asp)

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We have recently carried out three surveys for abnormal haemoglobins in Iran. Two of them were made in 1964 and in 1965, respectively, at the University Hospital in Shiraz. On both occasions 100 hospital patients coming from Shiraz and near-by areas were investigated, none of whom suffered from haemolytic anaemia. On examination of their haemoglobins for signs of thalassaemia and for abnormal haemoglobins two cases of haemoglobin A+D were found, and in both the D variant was identified by the "fingerprint" technique as haemoglobin D Punjab.

A third survey was carried out in Teheran of 400 consecutive patients presenting with anaemia. Of these, 70 were shown to have thalassaemia, three sickle-cell thalassaemia, 17 the sickle-cell trait, one sickle-cell haemoglobin D disease, and one haemoglobin C disease. In one patient a fast-moving haemoglobin was discovered. All the patients showing sickling came originally from the south coast of the Caspian Sea, but the fast-moving haemoglobin was found in a man of Central Iranian stock.

Haemoglobin J Iran

The fast-moving haemoglobin had the electrophoretic mobility of haemoglobin J—that is, on paper electrophoresis at pH 8.9 it moved faster than haemoglobin K but more slowly than haemoglobin N. In a family study this haemoglobin J was found in three generations, and it was inherited as a Mendelian codominant character (Fig. 1). The haematological examination of the father of the propositus, who also was a haemoglobin J trait carrier, gave normal haematological results. Haemoglobin 14.2 g./100 ml.; red cells 4,900,000/cu. mm.; packed cell volume 41%; M.C.V. 84 cu.- μ ; M.C.H. 31 μ g.; M.C.H.C. 35%. No target cells or any other abnormal red cells were seen in the peripheral blood smear. Thus this haemoglobin variant by itself does not cause anaemia, and the cause of the anaemia in the propositus remains to be determined.

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Chemical Investigation of Haemoglobin J Iran

Haemoglobin solutions were prepared and electrophoresis was carried out on paper, cellulose acetate, starch gel, and in starch block by the usual methods. On all occasions it was noted that haemoglobin A and haemoglobin J were present in about equal quantity (Fig. 2). Haemoglobin A consists of two α and two polypeptide chains, and its formula can be written $\alpha_2\beta_2$. Haemoglobin J moves faster than haemoglobin A towards the

FIG. 1.—Pedigree of Iranian family with Haemoglobin J Iran. ○ Haemoglobin A only. ● Haemoglobin A+J.

FIG. 2.—Starch-gel electrophoresis of the haemoglobin of the propositus (left) and a Haemoglobin A control. Haemoglobins A and J are present in equal quantity in this haemoglobin of the propositus.

positive pole on electrophoresis at alkaline pH, and this indicates a greater negative charge. To determine whether the difference in charge rested in the α - or in the β -chains, pure haemoglobin J was prepared by starch block electrophoresis. The haem groups were removed and the globin was compared with globin from haemoglobin A by the method of Chernoff and Pettit (1965). This technique involves the electrophoresis of globin in starch gel at pH 8 in the presence of 6 molar urea. At this high concentration of urea the α - and β -chains of the globin become dissociated, and on electrophoresis the α -chains move towards the cathode and the β -chains move towards the anode.

It could be seen that the α -chains of the globin from haemoglobin J moved exactly like the α -chains from the globin of haemoglobin A, but that the β -chains of the globin from haemoglobin J moved faster towards the anode than those derived from haemoglobin A (Fig. 3). This indicated that

FIG. 3.—Separation of peptide chains; starch gel pH 8. (1) Haemoglobin A; (2) Haemoglobins A+J Iran. An additional β -chain is present in "2"; this chain moves faster towards the anode than the β A chain, indicating a greater negative charge.

the increased negative charge of the haemoglobin J was situated in its β -chains, and that haemoglobin J was a $J\beta-(\alpha_2\beta_2^J)$. This finding was confirmed by hybridization of the isolated haemoglobin J with canine haemoglobin. Hybridization of normal adult human haemoglobin (haemoglobin A) with canine haemoglobin was carried out at the same time as a control (Itano and Robinson, 1953; Huehns, Shooter, and Beaven, 1962). After dissociation of the haemoglobin mixtures and recombination, the hybrids of human α -chains from both haemoglobins A and J with canine β -chains did not differ in their electrophoretic properties. However, when the hybrid $\alpha_2^{\text{canine}}\beta_2^A$ was compared with $\beta_2^{\text{canine}}\beta_2^J$ by electrophoresis in starch gel at pH 8.6 it was noted that $\alpha_2^{\text{canine}}\beta_2^J$ hybrid moved faster towards the anode—that is, it carried a greater negative charge than the $\alpha_2^{\text{canine}}\beta_2^A$ hybrid.

To discover whether the haemoglobin J β was a new variant or perhaps haemoglobin J β Baltimore (Baglioni and Weatherall, 1963), its globin was subjected to tryptic digestion, and the soluble tryptic peptides were separated by electrophoresis at pH 6.5 in one direction and by ascending chromatography in another. Thus a "fingerprint" or peptide map of the soluble tryptic peptides of the haemoglobin J was obtained (Fig. 5). At the same time a fingerprint of haemoglobin A was prepared (Fig. 4). The methods used were similar to those of Ingram (1958) and Baglioni (1961). Full details of the various techniques have recently been summarized by Watson-Williams, Beale, Irvine, and Lehmann (1965). When the two fingerprints (Figs. 4 and 5) are compared it will be seen that

two peptides in the fingerprint of haemoglobin A (numbers 1 and 2 in Fig. 4) are missing from that of haemoglobin J, but that two new peptides (3 and 4) can be seen (Fig. 5). Below the Number 4 peptide another additional peptide can be seen (marked by a star). This peptide gave a positive reaction for tyrosine; it often appears in fingerprints, and is the result of some chymotryptic digestion of the tryptic peptide of the β -chain which comprises residues 121–132 of its 146 amino-acid residues (β TpXIII). The chymotryptic peptide represents residues 121–130 of the β -chain and contains a tyrosine residue

FIG. 4.—Peptide chromatogram ("fingerprint") of Haemoglobin A. 1 indicates β TpIX (amino-acid sequence 67–82 of the β A chain); 2 indicates β VIII-IX (amino-acid sequence 66–82 of the β A chain).

FIG. 5.—Peptide chromatogram ("fingerprint") of Haemoglobin J Iran. 1 and 2 indicate where β TpIX and β TpVIII-IX are missing. 3 and 4 indicate the peptides replacing them. For details of these peptides and also of the one marked by a star see text.

situated in position 130. This third (starred) peptide can therefore be ignored, and the new peptides 3 and 4 in the fingerprint of haemoglobin J presumably represented the difference between haemoglobins A and J. The two peptides 1 and 2 in haemoglobin A correspond to the tryptic peptides IX and VIII-IX of the β -chain. β TpVIII by itself is not a true peptide but is free lysine, and in the intact β -chain it represents the 66th of its 146 residues. The tryptic cleavage between this lysine residue and the subsequent 16 residues 67–82 which form β TpIX is not complete. As a result free lysine (β TpVIII) and β TpIX as well as β TpVIII-IX are all three present in the fingerprint of haemoglobin A.

β TpIX contains histidine, and, on staining the fingerprints for histidine, peptides 1 and 2 in the fingerprint of haemoglobin A gave the expected positive reaction, but there was no histidine in the peptides 3 and 4 of haemoglobin J. As haemoglobin J is a "fast-moving" haemoglobin with a greater negative charge than haemoglobin A, it was possible at this stage to speculate that the histidine residue in position 77 of the β -chain in haemoglobin A had been replaced by the residue

of a negatively charged amino-acid, either glutamic acid or aspartic acid.

The genetic code which gives the base sequence of nucleotide triplets (codons) of messenger ribonucleic acid controlling the incorporation of amino-acids into proteins has been worked out mainly from the study of bacterial systems (Nirenberg et al., 1965). However, presuming that the allelic human haemoglobin variants are the outcome of single point mutations (the alteration of one base in a codon) it has been shown that, so far, they all obey the genetic code (Beale and Lehmann, 1965).

Accordingly the bases of the nucleotides coding for histidine are cytosine-adenine-cytosine or cytosine-adenine-uracil (CAC or CAU) and those coding for glutamic acid are GAA or GAG (G=guanine). No single alteration in the codons for histidine can convert either of them into a codon for glutamic acid. The triplet codons for aspartic acid, however, are GAC or GAU, so that a single alteration of the first base of the histidine codons from C to G would convert them into codons for aspartic acid.

Thus, based merely on the staining for histidine of the two fingerprints for haemoglobin A and J, one could expect the new variant to be the outcome of a replacement of $\beta 77$ His, the only histidine residue in β^A TpIX, by a residue of aspartic acid.

Peptide 3 was cut out from the fingerprint of haemoglobin J and eluted with 6N hydrochloric acid according to Sanger and Tuppy (1951) and Clegg, Naughton, and Weatherall (1965). After hydrolysis the amino-acids were estimated by automatic analysis according to Spackman, Stein, and Moore (1958), and the result was compared with the analysis of peptide 1 (β^A TpIX) from the fingerprint of haemoglobin A. Table I shows that peptide 3 is β^J TpIX, which differs from

TABLE I.—Amino-acid Analysis of β TpIX (Amino-acid Sequence 67–82 of the β -chain) of Haemoglobin A and of Haemoglobin J Iran

Molar Ratio of Amino-acid Residues			
Residues		β^A TpIX	β^J TpIX
Asp		2.7	3.7
Ser		1.2	1.5
Gly		2.0	2.1
Ala		2.4	2.1
Val		1.1	1.0
Leu		3.7	3.6
Phe		1.0	1.0
Lys		0.9	1.0
His		1.1	0

The asparagine residue in position 80 would on acid hydrolysis become aspartic acid.

β^A TpIX by having an aspartic acid residue instead of a histidine residue. The asparagine residue in position 80 is converted into aspartic acid on acid hydrolysis and appears as such in the analysis of both β^A TpIX and β^J TpIX. If we assume that there has been no change in the sequence of the other amino-acid residues, the formula of haemoglobin J Iran can therefore be written as $\alpha_2\beta_2$ 77 His-Asp (Table II).

TABLE II.—Amino-acid Sequence of β^A TpIX (Residues 67–82 of the β^A -chain) and Presumed Sequence of β^J IranTpIX

	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
A	Val	Leu	Gly	Ala	Phe	Ser	Asp	Gly	Leu	Ala	His	Leu	Asp	Asn	Leu	Lys
J Iran	Val	Leu	Gly	Ala	Phe	Ser	Asp	Gly	Leu	Ala	Asp	Leu	Asp	Asn	Leu	Lys

Electrophoretic Mobility at pH 8.6 and pH 6.5

The two new peptides 3 and 4 in the fingerprint of haemoglobin J are nearer the cathode than β^A TpVIII–IX and β^A TpIX respectively (Figs. 4 and 5)—that is, they are more negatively charged. Haemoglobin J β Iran has an electrophoretic mobility at pH 8.6, which indicates the presence of one additional negative charge per β -chain. The difference in electrophoretic mobility of haemoglobin J peptides 3 and 4 at pH 6.5 from that of the haemoglobin A peptides 1 and 2 is, however, greater than would be expected had they differed

by one charge only—for example, if a neutral residue had been replaced by an acidic residue. The observed mobility is more consistent with that to be expected if a partially positively-charged histidine residue had been replaced by an acidic residue. Histidine carries no detectable charge at pH 8.6 and would behave like a neutral residue at that pH. Its replacement by an acidic residue would result in an electrophoretic mobility associated with haemoglobin J, which differs from haemoglobin A by one additional negative charge per half-molecule. However, at pH 6.5, at which electrophoresis of the tryptic peptides is carried out, histidine is partially positively charged, and the substitution of a histidyl residue by an acidic residue would cause a greater difference between β^A TpIX and β^J TpIX and β^A TpVIII–IX and β^J TpVIII–IX, respectively, than would be expected from the difference in the electrophoretic mobility of the haemoglobins at pH 8.6.

It is of interest to compare the fingerprint of our haemoglobin J with that obtained from haemoglobin Seattle (Huehns and Shooter, 1965). In haemoglobin Seattle a neutral alanine residue in position 70 or 76 of the β -chain is replaced by one of glutamic acid. The resulting β^{Seattle} TpIX moves less towards the positive pole than β^J TpV. β^J IranTpIX (peptide 3) moved, however, further towards the anode than β^J TpV, which is the deeply staining peptide seen immediately below. The explanation is of course that at pH 6.5 β^J TpIX ($\beta 41$ –59) from haemoglobin J Iran (77 His→Asp) is more negatively charged than the corresponding peptide from haemoglobin Seattle (70 or 76 Ala→Glu).

There has been in the past some contradiction about the electrophoretic and chromatographic mobility of haemoglobin J at acid pH. Some authors—for example, Ager and Lehmann (1958)—reported that haemoglobin A+J did not separate on electrophoresis at pH 6.5 or at IRC 50 chromatography at pH 6; others obtained a good separation—for example, V. Minnich, personal communication. The explanation may well be found in their having used different haemoglobins J, the latter authors perhaps studying one similar to haemoglobin J Iran, when at pH 6.5 the absence of a histidine residue would

FIG. 6.—Electrophoresis at pH 8.9 of Haemoglobins A+J Baltimore ($\beta 16$ Gly→Asp) (left) and Haemoglobins A+J Iran ($\beta 77$ His→Asp) (right). At this pH the separation between A and J is the same in both samples.

FIG. 7.—Electrophoresis at pH 6.5 of Haemoglobins A+J Baltimore (left) and A+J Iran (right). At this pH histidine becomes partially charged. The absence of one histidine residue in J Iran allows, therefore, a separation from A, whereas A and J Baltimore, which both contain the same $\beta 77$ histidine residue, do not separate.

cause a considerable difference in electrophoretic mobility when compared with haemoglobin A (Figs. 6 and 7).

Summary

In two surveys, each of 100 non-anaemic patients in Shiraz, Iran, two examples of haemoglobin D Punjab were found. In a third survey of 400 anaemic patients in Teheran, thalassaemia, haemoglobin S, haemoglobin D, and haemoglobin C were found. In addition, a new haemoglobin, J Iran, $\alpha_2\beta_2$ 77 His→Asp, was discovered.

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Medical Memoranda

Massive Haematuria Associated with Sick-cell Trait

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The incidence of sickle-cell disease in the Negro race in the United States has been estimated at between 7% (Killingsworth and Wallace, 1936) and 13% (Beck and Hertz, 1935). Among West Indian Negroes in Jamaica the incidence of this disease has been estimated at 11% by Went (1957). The incidence of the heterozygous sickle-cell trait (haemoglobinopathy A-S) is estimated as 40 times that of the homozygous sickle-cell anaemia (haemoglobinopathy S-S).

As a result of immigration, sickle-cell disease is now often encountered in Britain (Dixon, 1962; Beard and Signy, 1965). Massive haematuria has become a well-established though rare complication of sickle-cell trait in the United States and the West Indies, but it does not seem to have been reported previously in Britain. The following case is therefore presented to draw attention to this condition and to suggest that this diagnosis should be considered early in the investigation of massive haematuria in a susceptible patient.

CASE REPORT

A 25-year-old Jamaican Negro was referred to hospital in December 1960 with profuse painless haematuria. There was nothing of note in either his previous medical history or his family history. No abnormal features were found on a routine examination of blood smears. An excretory pyelogram was normal. Cystoscopy was undertaken on two occasions and no cause for the bleeding was found. Ascending pyelograms were performed; on the right side the appearances were normal, while there was a filling defect in the upper calix on the left side. A tumour was suspected, but an aortogram did not confirm this diagnosis.

Massive haematuria recurred on 10 April 1961 and persisted after admission to hospital. The left kidney was explored after cystourethroscopy on 26 April. The kidney appeared normal to palpation and it was possible to invaginate the pelvis into the suspicious upper calix without any abnormality being detected. In the post-operative period haematuria recurred.

A second urethroscopy was undertaken on 18 May in another hospital. Granulation tissue was found in the posterior urethra and diathermy was given. Several specimens of urine were found to be free of red cells at this time, and the possibility of a self-inflicted injury was considered.

He was next referred to the Urological Clinic at the General Hospital, Birmingham, on 7 July. At this time he had a heavy haematuria, a blood-pressure of 180/115 mm. Hg, haemoglobin 12.7 g./100 ml., and serum urea 26 mg./100 ml. A mid-stream specimen of urine contained a significant number of pus cells and yielded *Escherichia coli* and *Proteus mirabilis* on culture. An excretory pyelogram showed a central translucency in the upper group of calices on the left side, thought to be blood clot.

He was admitted to the Queen Elizabeth Hospital, Birmingham, on 3 November on account of further persistent haematuria associated with pain and discomfort in the left renal area. Haemoglobin was then 8.8 g./100 ml., and increased variation in the size and shape of the red cells was noted. Serum urea was 22 mg./100 ml. and the urine was sterile although profuse numbers of red cells were reported. Blood-pressure remained 180/115 mm. Hg.

At cystoscopy on 6 November blood was seen coming from the left ureteric orifice. A left ascending pyelogram was performed, and as this showed a constant filling defect the left kidney was explored later the same day. There were dense adhesions from the previous exploration and the kidney was mobilized only with difficulty. The ureter appeared normal apart from containing blood clot; the kidney also appeared normal but was difficult to examine. In view of the protracted bleeding from this kidney a nephrectomy was performed.

The kidney weighed 105 g. and seemed to be normal apart from a depressed area measuring 2 by 2 cm. at the upper pole, to which the capsule was adherent and the borders of which were haemorrhagic. On histological examination there was an area of dense fibrosis with loss of tubules and crowding together of glomeruli, which themselves showed loss of structure but no dense fibrosis. Around the periphery of the lesion was a chronic inflammatory reaction consisting mainly of lymphocytes. Occasional haemosiderin-laden macrophages and haematoidin crystals were present. A small artery was occluded by thrombus. While the nature of this lesion remained obscure, the appearances most closely resembled those of infarction, but were by no means typical. The remainder of the kidney showed no histological abnormality.

He made an uneventful recovery from the nephrectomy and was finally discharged on 3 January 1962, there having been no further haematuria.