Haemoglobin J Tongariki ($\alpha 115$ Alanine $\rightarrow$ Aspartic Acid): the first new haemoglobin variant found in a Pacific (Melanesian) population

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In the course of a medico-genetic-anthropological investigation in 1963 of the population of the island of Tongariki, in the Shepherd group of the New Hebrides, a haemoglobin variant was detected with a mobility which was greater than that of normal Hb on electrophoresis at alkaline pH, both by starch gel and by paper electrophoresis.

The variant was found in this first survey in 17 of 228 persons from whom blood was obtained for electrophoresis. Of these, 15 were contained in one pedigree and no departures from simple Mendelian inheritance were noted. The pedigree also indicates that the gene must have been present on the island at least four generations ago.

The population on the island of Tongariki is predominantly Melanesian, with little marriage outside the island (Buninga; Mweriu, and Mangarisu villages on Tongoa) but with admixture of a small Samoan element and also of Australian aborigines. Pedigree analysis suggests that the present haemoglobin variant is independent of either of these two extraneous elements in the population.

Blood samples were collected twice, in 1963 and in 1965. On the first occasion they were collected into Bayer venules and immediately placed into a portable refrigerator. They were then transported by ship and air and arrived in Perth (Australia) 48–72 hours after collection. On a second visit in 1965 fresh samples were obtained and transported on wet ice to Melbourne, where the clots were

separated and placed into liquid nitrogen. They reached Cambridge, after being first sent to Washington, but were still cold on arrival.

From some of the first samples blood films were prepared, and no abnormal red cells or any other abnormalities were noted, which could be associated with the haemoglobin variant. This examination was kindly performed by Professor R. W. Pitney, Haematology Department, University of New South Wales.

Preparation of Haemoglobin

The second collection of samples consisted of about 300 clots of blood which had been frozen in transit: thus the cells were lysed, and it was not possible to wash these in isotonic saline to remove serum protein. A fair number of tubes were broken in the course of the flight from Melbourne to Washington and from Washington to London. Of the 300 specimens, 289 were recovered for further investigation. The samples were centrifuged and the supernatant was examined by paper electrophoresis using Tris buffer, pH 8.9, for the Hb J variant. Eleven specimens showed two Hb bands, one in the position of Hb A, and one in that of Hb J. Fig. 1 shows two samples with Hb A and J.

To prepare a pure solution of J, the band with Hb J was cut out and eluted with water. The eluate was then concentrated *in vacuo* until the Hb concentration was approximately 10%. As several serum proteins have at pH 8.9 the same electrophoretic mobility as the fast-moving Hb found in the Tongariki specimens, further purification was required. The Hb solution was added to a saturated ammonium sulphate solution 4.5 : 5.5 v/v and left to stand overnight. The

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FIG. 1. Electrophoresis at pH 8.9 of two specimens. Solutions obtained from frozen blood show two bands of haemoglobin, one in the position of Hb A, and one moving faster in the position of Hb J.

precipitated serum proteins were removed by centrifuging, and the supernatant was dialysed against distilled water and concentrated *in vacuo* to remove the ammonium sulphate. When the concentration of the Hb was again 10%, the ammonium sulphate precipitation was repeated, and again the precipitate and the ammonium sulphate were removed as before. This was repeated until no more precipitate appeared on standing overnight with a 4.5:5.5 v/v saturation of ammonium sulphate. The 10% Hb solution was then again submitted to paper electrophoresis to separate any remaining traces of Hb A. The fast-moving Hb band was cut out and eluted with water and the eluate concentrated until the haemoglobin concentration was 10%. Some of the Hb was submitted to this procedure until all Hb A had been removed. Such solutions were used for the hybridization procedures and for the electrophoresis of the separated α - and β -chains in starch gel with 6 M urea, and also for the original fingerprints (peptide chromatograms). As, however, this repeated purification involved a considerable loss of the fast-moving haemoglobin, most of the work on the amino acid sequence of this haemoglobin was carried out on preparations which also contained some Hb A. As this work was done with separated peptide-chains, those of Hb A were removed automatically during chain separation.

Variant Peptide Chain

To find out whether the Hb was abnormal in its α - or its β -chain a solution of pure Hb J was dissociated and the dissociated chains were then recombined in the presence of canine Hb. Even before hybridization with canine Hb it seemed likely that the abnormality in the Hb J Tongariki rested in its α -chain, because both on paper

electrophoresis at pH 8.9 (Cradock-Watson, Fenton and Lehmann, 1959) and on starch gel electrophoresis at pH 8.3 (Poulik, 1957), two Hb A bands had been noted, indicating that there were present Hb $\alpha_2^A\delta_2$ and $\alpha_2^J\delta_2$. On hybridization with canine Hb (Itano and Robinson, 1959; Huehns, Shooter, and Beaven, 1962), the canine plus β_2 J Tongariki hybrid had the same mobility as the hybrid formed by the canine α_2 subunits with the β_2 subunits of Hb A. However, the hybrids of the β_2 subunits of canine Hb with the α_2 subunits of Hb A and the α_2 subunits of Hb J Tongariki, respectively, differed in their electrophoretic mobility, the Hb J Tongariki hybrid moving faster towards the anode on electrophoresis at pH 8.3. This observation confirmed that Hb J Tongariki was an α -chain variant, a Hb J α . Further confirmation was obtained when the globins of Hb J and A were submitted to starch gel electrophoresis at pH 8.0 in the presence of 6 M urea (Chernoff and Pettit, 1964). As well as the expected α - and β -chains of Hb A, a β -chain band with the electrophoretic mobility of the β^A -chain was found in the globin of Hb J Tongariki, but the α -chain band had moved considerably faster towards the anode than the α -chain of Hb A. This indicated a difference in charge between α^A and α^J , and as Hb J, in contrast to Hb I or N, carries one additional negative charge rather than two, the likely mutation in Hb J Tongariki was either the replacement of a lysine or arginine residue by a neutral residue, or the replacement of neutral amino acid residue by one of glutamine or aspartic acid.

Amino Acid Substitution in Hb J Tongariki

Fingerprint of Hb J Tongariki. The globin was prepared and its tryptic digestion, routine fingerprints, and colour tests for specific amino acid residues were performed as summarized in 1967 by Watson-Williams, Beale, Irvine, and Lehmann. These procedures showed the soluble tryptic peptides to have the same electrophoretic and chromatographic mobilities and the same specific colour reactions as those of Hb A.

Of the 141 residues of the α -chain, some do not appear in the fingerprint of the soluble peptides: residues 100-139, comprising the tryptic peptides α XII (residues 100-127) and α XIII (128-139) are largely insoluble on tryptic hydrolysis. As the fingerprint of the soluble tryptic peptides of Hb J Tongariki was indistinguishable from that of Hb A, it was likely that the amino acid substitution had occurred in the so-called 'core' of the α -chain, i.e. amongst residues 100-139.

Preparative fingerprints were made using 4 mg. of the α -chains from Hb J Tongariki, and a Hb A control. The material was applied to a 1.5 cm. starting line for electrophoresis, and the band width subsequently reduced by washing in with 2% formic acid before chromatography. From the resulting fingerprints the peptides under discussion were eluted, and after hydrolysis in 6N HCl at 105°C. for 22 hours amino acid analysis was carried out. The results are shown in Table I. They show that the eluted peptide is α TpXIIB, i.e. residues 105-127, and that the difference between the peptides from Hb A and Hb J Tongariki consists in a replacement of an alanine residue in Hb A by one of aspartic acid in Hb J Tongariki.

Using Jones' technique in conjunction with standard tryptic hydrolysis and peptide fingerprinting, Clegg, Naughton, and Weatherall (1966) showed that of the α -chain core the portion 100-104 could be clearly seen on the fingerprint of the AE α -chain, but that the rest of the core was represented inconclusively by a smear at the origin.

Using the techniques described by Clegg, Naughton, and Weatherall (1965), the α -chains of Hb J Tongariki were separated by an ionic concentration gradient on a carboxy methyl cellulose column in the presence of 8 M urea and mercaptoethanol. Fig. 2 shows the chromatogram obtained by monitoring the 280 m μ absorption

FIG. 2. Curves obtained from monitoring at 280 m μ the effluent of a chromatographic column (for details see text) which separates the α - and β -chains of a haemoglobin sample containing mainly Hb J Tongariki, but also some Hb A. Only one type of β -chains, those of Hb A, are obtained, but two types of α -chains: α^A and α^J , the α^J chains having a greater negative charge.

TABLE I
AMINO ACID ANALYSIS OF TRYPTIC PEPTIDE α TpXIIb
FROM Hb I TONGARIKI AND Hb A

	Hb Tongariki		Hb A		
	μmoles	Residues	μmoles	Residues	Expected
Aspartic	0·030	2·0	0·055	1·2	1
Threonine	0·030	2·0	0·095	2·2	2
Serine	0·020	1·3	0·063	1·4	1
Glutamic	0·014	1·0	0·045	1·0	1
Proline	0·021	1·4	0·065	1·5	2
Alanine	0·057	3·8	0·205	4·7	5
Valine	0·033	2·2	0·091	2·0	2
Leucine	0·068	4·5	0·181	4·1	5
Phenylalanine	0·015	1·0	0·046	1·0	1

No analysis of basic residues was made.

Amino Acid Sequence of α TpXIIb of Hb A

105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127
 Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-Pro-Ala-Glu-Phe-Thr-Pro-Ala-Val-His-Ala-Ser-Leu-Asp-Lys

Arrows indicate some possible points of chymotryptic cleavage.

Chymotryptic Digestion of Tryptic Peptide α 105-127 (α XIIb). As demonstrated in Table I, one of the five alanine residues of peptide TpXIIb of the α -chain of Hb A has been replaced by an aspartic acid residue in Hb J Tongariki. The five alanine residues are in positions 110, 111, 115, 120, and 123. It was not possible to obtain enough material from the digests of the AE α -chains of Hb A and Hb J Tongariki to embark on a further analysis of the α TpXIIb peptides, and thus to determine which of the five alanine residues had been substituted. It was concluded that the bulk of the α -chain core peptides was present in the material that was still insoluble at neutral pH even after extended tryptic hydrolysis under the standard conditions. This amounted to about one-quarter of the original material. This insoluble material was separated from the supernatant and washed twice

with pH 6.4 buffer which had been prepared by adding 0.5 M acetic acid drop-wise to 0.1 M ammonium bicarbonate. This washed material was then suspended in ammonium bicarbonate buffer pH 6.5 and digested with chymotrypsin at 37°C. for 2 hours.

Fingerprints were prepared of the chymotryptic peptides using the same electrophoretic (pH 6.5) and chromatographic techniques as for the tryptic peptides. A clear difference was seen between the fingerprints obtained from the Hb J Tongariki material and from the Hb A control. As shown in Fig. 4, two peptides noted in the fingerprint of the Hb A material were missing from that of the material from Hb J Tongariki, and a new peptide had appeared which was not present in the control. This extra peptide gave a strong reaction for histidine and ran just to the anode side of the neutral area.

Amino acid analysis of this peptide from a

FIG. 3. Fingerprint of the AE α -chain of Hb J Tongariki (electrophoresis at pH 3.5). α TpXIIb is missing and a new peptide with a greater negative charge and lower chromatographic mobility can be seen instead.

FIG. 4. Fingerprint of the chymotryptic peptides of the tryptic core of the AE α -chain of Hb J Tongariki. No chymotryptic split at bond 117-118 has occurred. The chymotryptic peptides 110-117 and 118-122 which are found in the Hb A control are missing here, and a peptide not found in the control is found moving slightly towards the anode - it represents residues 110-122 of the α -chain of Hb J Tongariki.

Chymotryptic peptides from insoluble material of AE α -chains of Hb A

110 111 112 113 114 115 116 117 118 119 120 121 122

Ala - Ala - His - Leu - Pro - Ala - Glu - Phe Thr - Pro - Ala - Val - His
↑

preparative fingerprint (Table II) showed that it comprised residues 110-122 of the α -chain with the replacement of an alanine by an aspartic acid. Thus, one of the five alanine residues of α ATpXIIb, namely alanine 123, could be eliminated as the possible point of mutation Ala $\rightarrow$ Asp in Hb J Tongariki. The equivalents of the two missing peptides on the control were also similarly analysed. The fast-running peptide was shown to be peptide α 118-122, and the neutral peptide was shown to be α 110-117 (see above).

TABLE II

AMINO ACID ANALYSIS OF CHYMOTRYPTIC PEPTIDES FROM THE ALPHA CORE OF Hb J TONGARIKI AND Hb A

	Alpha Tongariki 110-122		Alpha A 110-117 and 118-122			
	μ moles	Residues	μ moles		Residues	Expected
			110- 117 (see below)	118- 122		
Aspartic	0.032	1.2			—	—
Threonine	0.024	0.9		0.067	1.1	1
Glutamic	0.027	1.0	0.057		0.9	1
Proline	0.045	1.7	0.060	0.061	2.1	2
Alanine	0.082	3.0	0.192	0.068	4.1	4
Valine	0.021	0.8		0.049	0.8	1
Leucine	0.031	1.1	0.077		1.2	1
Phenylalanine	0.024	0.9	0.069		1.1	1

	Alpha Tongariki 110-117		Alpha A 110-117		Expected
Aspartic	0.045	1.1	—	—	—
Glutamic	0.042	1.0	0.057		1
Proline	0.039	1.0	0.060		1
Alanine	0.076	1.9	0.192		3
Leucine	0.041	1.0	0.077		1
Phenylalanine	0.036	0.9	0.069		1
Histidine*	*	1	*		1

* A positive specific colour test was considered to represent one residue.

It was therefore clear that the Hb J Tongariki material had not been hydrolysed by chymotrypsin between residues 117 and 118. The most probable explanation was that the substitution in Hb J Tongariki exerted an inhibition on the chymotryptic splitting of the peptide bond formed by phenylalanine 117 with threonine 118, and that this substitution was one of aspartic acid for alanine in position 115, the combined negative charges 115 Asp-116 Glu inhibiting chymotryptic cleavage at Phe 117-Thr 118.

On increasing chymotryptic digestion time from 2 hours to 4½ hours a break could be obtained at Phe 117 in the Hb J Tongariki digest. With this procedure the fast-moving peptide α 118-122 was obtained in the same position as in the control, and a new negatively-charged peptide appeared which on amino acid analysis (Table II) was found to be α 110-117, with an aspartic acid residue replacing an alanine. Thus, of the four remaining possible points of substitution, α 120 was eliminated and only the alanine residues 110, 111, and 115 remained, of which Ala 115 was the most likely to have been substituted by a residue of aspartic acid.

Digestion of Chymotryptic Peptide α 110-117 with Carboxypeptidase. Peptide 110-117 from the AE α -chain of Hb J Tongariki was eluted from an electrophoretic paper strip which had been prepared using the chymotryptic digest of the residue from the tryptic digest of 12 mg. of AE α -chains, the yield being estimated at 0.1/0.3 μ mol. One half of this was subjected to prolonged digestion with carboxy-peptidase A, which removes sequentially the carboxy terminal residues of peptides, but is blocked by proline residues. The lyophilized digest was run electrophoretically as a 1 cm. band at pH 3.5, 3 Kv, for 40 minutes side by side, with marker amino acids, an enzyme blank, and an enzyme and paper blank. When this electrophoretogram was stained with ninhydrin it showed a dense phenylalanine band, a somewhat less dense glutamic acid band, and a faint but definite aspartic acid band. No aspartic acid was seen in the controls. It was concluded that in Hb J Tongariki an alanine residue in position 115 of the α -chain is replaced by one of aspartic acid (Fig. 5).

FIG. 5. The peptide contains two alanine residues and one aspartic acid residue. To determine the position of these three residues the peptide was examined by stepwise degradation from the amino end and by the action of carboxypeptidase attacking the carboxy end. By this means it was possible to demonstrate that residues 110 and 111 were alanyl and that residue 115 was aspartyl.

Degradation of Chymotryptic Peptide α 110–117 from N Terminal. Additional confirmation was obtained using the second half of the peptide for analysis by the Dansyl-Edman technique (Gray and Hartley, 1963), which sequentially identifies amino-terminal residues. A two-stage procedure using this technique indicated an amino-terminal sequence Ala-Ala for the peptide α 110–117 from Hb J Tongariki. The sequence can thus be written as follows:

110 111 112 113 114 115 116 117
Ala – Ala – His – Leu – Pro – Asp – Glu – Phe

Hb J Tongariki has not been described in any other population, and it will be of interest to see whether it is unique for the island of Tongariki and people derived therefrom, or whether it will be found in other surveys of Melanesian populations.

Summary

In 17 of 228 blood samples from inhabitants of Tongariki, an island in the Shepherd group of the New Hebrides, a haemoglobin variant was found with the mobility of Hb J. Pedigrees indicated that it must have been on the island at least four generations ago, and that it could be associated with the Melanesian element. Its chemical investigation presented interesting new problems, because the difference of this haemoglobin from normal

adult Hb A was situated in the α -chain core, i.e. a part of the haemoglobin molecule which has not yet been examined in detail for haemoglobin variants. Hb J Tongariki was found to be $\alpha_2^{115\text{Ala} \rightarrow \text{Asp}}\beta_2$.

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