HEMOGLOBIN CASTILLA β 32 (B14) Leu → Arg; A NEW UNSTABLE VARIANT PRODUCING SEVERE HEMOLYTIC DISEASE

M. C. GAREL, Y. BLOUQUIT and J. ROSA
With the technical assistance of N. AROUS
Unité de Recherches sur les Anémies I.N.S.E.R.M.U 91, Hôpital Henri Mondor 94010 Créteil, France

and

C. ROMERO GARCIA
CSSS La Paz Servicio de Hematologia, Madrid, Spain

Received 24 July 1975

1. Introduction

The unstable hemoglobins are a group of well defined hemoglobin variants associated clinically with haemolytic anaemia of varying severity. The nature of the amino acid substitutions reduces the stability of the hemoglobin molecule, resulting in precipitation within the red cell and formation of characteristic inclusions called 'Heinz bodies'. Most of these hemoglobins involve amino acid substitutions either at sites neighboring the heme group of the β polypeptide chain, or at the α_1 β_1 contact if the nature of the substitution weakens the bonds at the contact itself.

This report describes a new unstable hemoglobin, which has been observed in a Spanish woman. This new variant which has been identified as β 32 (B14) Leu → Arg, is an homologue of Hb Abraham-Lincoln [1], β 32 (B14) Leu → Pro an unstable variant. This instability arises from the substitution of a proline for a leucine residue, which produces a disruption of the normal orientation of the adjacent leucine residue at β 31 and impairs heme stabilization.

2. Materials and methods

Routine hematological examinations and enzyme assays were performed by standard methods [2] on fresh blood specimens. Hemolysates were prepared according to Drabkin [3]. Tests for heat stability, isopropanol solubility, and dissociation resulting from the reaction of hemoglobin with PHMB (*p*-hydroxy mercuribenzoate) were carried out as previously described [4]. Standard methods were employed for hemoglobin electrophoresis [5]. The abnormal β chain of the unstable hemoglobin variant was isolated by PHMB treatment [4]. Chains were separated by electrophoresis or by carboxy methyl cellulose (CM cellulose) chromatography in 8 M urea buffer [6]. Tryptic peptides were isolated by analytical and preparative finger prints on thin layer silica gel plates (Schleicher and Schüll) according to Braconnier [7], and their amino acid composition established on a Jeol JLC 5 AH amino acid analyser.

3. Results

3.1. *Case report*

This patient is a 25 year old woman who was first investigated at the age of 3 years as a result of recurrent attacks of severe jaundice associated with pallor. A splenectomy was performed when she was 4 years old. She has since had a variable degree of jaundice and anemia accompanied by dark urine. The manifestations have worsened strikingly with infections.

Neither parent of the propositus had been known to have anemia or jaundice. Blood samples from the parents showed normal hematological findings and neither showed evidence of anemia, erythrocyte inclusion body formation, or a heat-unstable hemoglobin fraction. The first child of the propositus is also normal.

3.2. *Hematological studies*

A blood sample from the patient showed these hematological values: hemoglobin: 9.6 g/100 ml, MCV: 110 μm^3, MCH: 34 pg, MCHC : 33%. The blood film showed anemia and moderate degrees of anisocytosis, and polychromasia. Her reticulocyte count was elevated. An osmotic study showed the fragility of her erythrocytes to be slightly diminished. All the erythrocyte enzymes activities were increased which is consistent with the elevated reticulocyte count. The percentage of methemoglobin was normal in fresh blood samples. Heinz bodies were seen in many cells and treatment with phenyl-hydrazine resulted in Heinz bodies being present in almost all. Other laboratory data included a serum iron of 109 μg/100 ml, an iron binding capacity of 472 μg/100 ml.

3.3. *Structural studies*

Electrophoresis of freshly-prepared hemolysates on cellulose acetate strips in Tris-EDTA-borate buffer at pH 8.6 demonstrated a band of hemoglobin

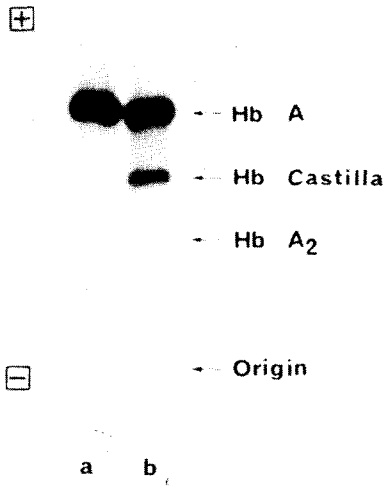

Fig.1. Electrophoresis on cellulose acetate strips of the patient's blood hemolysate (b) and of a normal hemolysate (a) in a Tris-EDTA-borate discontinuous buffer, pH 8.9.

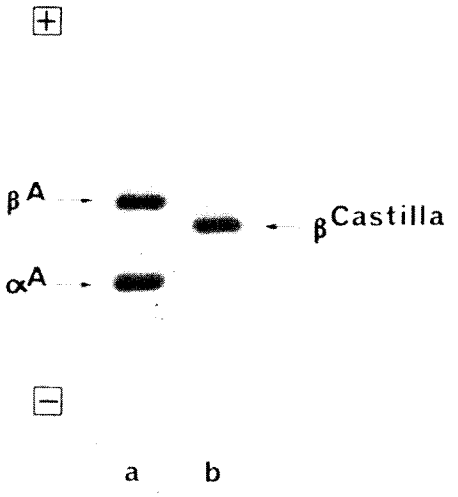

Fig.2. Electrophoresis on cellulose acetate strips of PHMB precipitated globin from the patient's hemolysate after 4 h of incubation in a discontinuous 6.0 M urea buffer, pH 7.0 (b). Control Hb A (a).

(22%) migrating more slowly than the major hemoglobin fraction which corresponded to hemoglobin A. The Hb A_2 level was normal (3.5%). Free α-chains formed a faint band near the origin (fig.1). When the hemolysate was heated at 50°C, a flocculent precipitate appeared which demonstrated heat lability of the hemoglobin. Instability was also demonstrated by isopropanol solubility test, since all the Hb Castilla was precipitated before 6 min.

Fig.2 illustrates the results of the isolation of the β chain from the precipitate which resulted from the treatment of total hemolysate with PHMB after 4 h of incubation. The material consisted mainly of β chains which migrate more slowly than normal β chains, together with small amounts of normal β and α chains. This abnormal β chain was isolated by CM-cellulose (CM52) column chromatography in 8 M urea buffer and converted into the aminoethylated derivative.

Fig.3 shows the finger print of the tryptic digest of the aminoethylated β chain from Hb Castilla. All of the expected peptides were present with the exception of the peptide β T4 which was missing. However 2 new peptides, (a) and (b), were present. One of them: peptide (a) stained only for arginine. The other, peptide (b) stained for tyrosine, tryptophan and arginine. The two abnormal peptides were eluted from

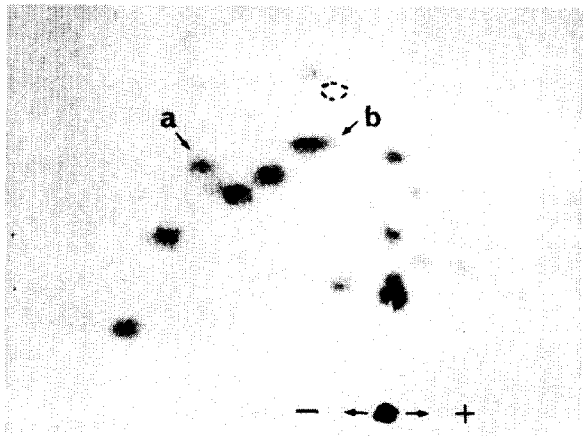

Fig.3. Map of the tryptic peptides of the aminoethylated and trypsin-digested β chains from Hb Castilla. Electrophoresis was carried out at pH 6.4 for 2 h and followed by ascending chromatography. The dotted circle indicates the position occupied by normal β T4. The arrows show the positions of the two supplementary peptides: ⓐ β T4a and ⓑ β T4b.

the preparative finger print with 6 N HCl and hydrolysed for 22 h at 110°C. Their amino acid compositions were determined on an automatic amino acid analyser (Jeol JLC 5 AH). Table 1 shows the amino acid contents of the two extra peptides ⓐ and ⓑ. These results indicate that in this variant the leucine residue 32 of the β chain has been replaced by an arginine. After tryptic digestion the arginyl peptide bond was cleaved, splitting the peptide β T4 into two new peptides, β T4a; Leu–Arg and β T4b; Val–Val–Tyr–Pro–Try–Thr–Gln–Arg.

4. Discussion

The hemoglobin abnormality identified in this patient is one of a number of variant Hb that produce hemolytic anaemia accompanied by the formation of inclusion bodies in the erythrocytes. All the amino acid substitutions found in the unstable hemoglobins have involved residues in the interior of the molecule.

Some substitutions occur either at an α β contact point, or are characterized by disturbances affecting the binding of heme to the globin chains. In another group of unstable Hb, the substitution of a polar amino acid for a non polar residue results also in molecular instability.

In the new variant described in this paper, the substitution of an arginine for a leucine at β 32 introduces a polar amino acid instead an apolar one in an internal position. This almost certainly explains the great instability of the molecule, since the B helix of the β subunits is distorted. This residue is apparently not in close contact with the heme group but distortion

Table 1
The amino acid composition of peptides β T4a and β T4b from β Castilla chain. The composition of normal β T4 is shown for comparison. Hydrolysis was carried out in vacuo with 6 M HCl, 0.009% phenol, 110°C, 22 h.

	β T4 (β 31–32)	β T4b (β 33–40)	Normal β T4 (β 31–40)
Residue	Molar ratio	Molar ratio	Expected molar radio
Arginine	0.95	0.90	1
Threonine		1.04	1
Glutamic acid		1.12	1
Proline		1.25	1
Valine		1.70[ab]	2
Leucine	0.70[b]	0	2
Tyrosine		0.81	1
Tryptophan		[ac]	1

[a] After 72 h hydrolysis
[b] N terminal residue can be extensively destroyed by ninhydrin
[c] Tryptophan detected on peptide maps by staining with Ehrlich reagent.

of the B helix could readily affect the heme contact with β 31 leucine and the α_1 β_1 contact with β 33 valine, β 34 valine and β 35 tyrosine.

Two variants, homologues of Hb Castilla, were described in which the leucine β 32 (B14) is replaced by a proline: Hb Perth [8] and Hb Abraham-Lincoln [1]. The replacement of an amino acid residue by a prolyl residue usually causes instability when the substitution occurs beyond the first three amino acid residues of an helical segment. The substitution in these cases occurs at the B14 position of the B helix and results in a redirection of the carboxy terminal part of this helix. This redirection disturbs contacts involving some amino acid residues of the B helix and causes instability of these variants.

The new variant described in this paper affords new evidence for the importance of the B helix in the stability of the β chain. Thus variants in B5 Hb Freiburg [9], B6 Hb Riverdale Bronx [10] and Savannah [11], B10 Hb St Louis [12] and Hb Genova [13], B12 Hb Tacoma [14] are very unstable hemoglobins.

Further studies will be necessary in order to determine the functional implication of the substitution. A more detailed study of this family is required in order to determine whether the substitution at β 32 (B14) Leu → Arg is a unique mutation.

Acknowledgements

This work was supported in part by l'Institut National de la Santé et de la Recherche Médicale, grant n° 73-5-037-2, by la Délégation Générale à la Recherche Scientifique et Technique, grant n° 72-7-028-2 and by la Fondation pour la Recherche Médicale Française.

The authors wish to acknowledge Dr John Chapman for careful assistance in reviewing the manuscript.

References

[1] Honig, G. R., Green, D., Shamsuddin, M., Vida, L. N., Mason, R. G., Gnarra, D. J. and Maurer, H. S. (1973) J. of Clin. Invest. 52, 1746–1755.
[2] Dacie, J. V. and Lewis, S. M. (1966) in: Practical Haematology, Vol. IV. (Churchill, ed.).
[3] Drabkin, D. L. (1949) Arch. Biochem. 21, 224–231.
[4] Carrel, R. W. and Kay, R. (1972) Brit. J. Haemat. 23, 615–619.
[5] Beuzard, Y., Courvalin, J. C., Cohen-Solal, M., Garel, M. C., Rosa, J., Gibaud, A. and Brizard, C. (1972) FEBS Lett. 27, 76–80.
[6] Clegg, J. B., Naughton, M. A. and Weatherall, D. J. (1966) J. Mol. Biol. 19, 91–108.
[7] Braconnier, F., El Gammal, H., Coquelet, M. L. Beuzard, Y., Garel, M. C. and Rosa, J. Nouv. Rev. Fr. Hemat., in press.
[8] Jackson, J. M., Yates, A. and Huehns, E. R. (1973) Brit. J. Haemat. 25, 607–610.
[9] Jones, R. T., Brimhall, B., Huisman, T. H. J., Kleihauer, E. and Betke, K. (1966) Science 154, 1024–1027.
[10] Ranney, H. M., Jacobs, A. S., Udem, L. and Zalusky, R. (1968) Biochem. Biophys. Res. Commun. 33, 1004–1011.
[11] Huisman, T. H. J., Brown, A. K., Efremov, G. D., Wilson, J. B., Reynolds, C. A., Uy, R. and Smith, L. L. (1971) J. Clin. Invest. 50, 650–659.
[12] Cohen-Solal, M., Seligmann, M., Thillet, J. and Rosa, J. (1973) FEBS Lett. 33, 31–41.
[13] Sansone, G. ,Carrell, R. W. and Lehmann, H. (1967) Nature, London 214, 877–879.
[14] Brimhall, B., Jones, R. T., Baur, E. W. and Motulsky, A. G. (1969) Biochem. 8, 2125–2129.