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IDENTIFICATION OF ABNORMAL HEMOGLOBIN KÖLN (β98 (FG-5) VAL→MET) : THE FOURTH HB KÖLN VARIANT FOUND IN JAPAN

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Abstracts

In 1977 an electrophoretically slow moving abnormal hemoglobin (6 per cent of total hemoglobin) was detected by cellulose acetate membrane electrophoresis (pH 8.6) from a 28 yr-old female with slight jaundice and splenomegaly.

Structural analyses of the hemoglobin demonstrated its identity with Hb Köln (β98 Val→Met). The results of physical, and hematological examination and functional studies of the hemoglobin were consistent with Hb Köln disease. The parents of the propositus were negative for Hb Köln, but a daughter (2 yr-old), only one her child was the carrier of the same abnormal hemoglobin. This family was the fourth independent Hb Köln disease described up to date in Japan.

INTRODUCTION

Congenital Heinz body anemias which are classified into the nonspherocytic hemolytic anemias[1] are characterized by hemolysis, hypersplenism and intraerythrocytic Heinz bodies owing to the presence of unstable hemoglobin in red cells. More than sixty subtypes have been reported from various districts of the world according as the difference in the structural difference of the molecules of the abnormal hemoglobins detected[2].

In 1963, Hb Ube-1 (β98 Val→Met)[3] was recorded by us as the first instance of unstable hemoglobin in Japan, but final establishment of amino acid substitution 10 years later disclosed that it was identical with Hb Köln which had been discovered in Germany[4]. Since then, the same hemoglobin has been reported from the Europeans[5,6] and from the Asiatics[7] and it is

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now generally recognized that this hemoglobin is classified as the most frequently encountered unstable hemoglobin of the world. In Japan, there are three papers to date describing Hb Köln disease without mutual kin relationship[8,9,10]. This paper will present the fourth family which has been confirmed by the abnormal hemoglobin analysis.

METHOD

Routine hematological and clinical chemistry examinations were carried out by standard methods. Conventional methods were employed for the preparation of hemolysate, and it was tested with respect to heat instability and isopropanol precipitation[11], isoelectric focusing on polyacrylamide gel with pH range of 7–9 ampholine[12] and Hb F content[13].

The abnormal hemoglobin was purified by cellulose acetate membrane electrophoresis using 0.05 M Tris–EDTA–borate buffer (pH 8.6). The band of abnormal hemoglobin on the membrane was cut out, eluted into 0.05 M Bis–tris buffer (pH 7.4). For the purpose of measurement of oxygen equilibrium curves, an aliquot of about 10 μM–Hb solution was adjusted to desired pH (7.0–7.4) by dialysis of the purified hemoglobin against 0.05M Bis–tris buffer solution in a cold room (4°C). Oxygen equilibrium was then measured by the method of Imai et al.[14].

The hemoglobin was examined for absorption spectrum in a Cary type 118C self recording spectrophotometer, and for chain anomaly by starch gel electrophoresis of preliminarily PCMB–treated hemolysate[15].

Structural analyses were performed in the following way: 1) Hemoglobin was converted to globin by the method of Anson and Mirsky[16]. 2) The α and the β chains of the globin were separated on a CM–cellulose column, using phosphate buffered 8M–urea solution as developer[17]. 3) The abnormal β chain thus obtained was aminoethylated, and digested with TPCK trypsin at 37°C and pH=8.0 for 3 hrs., followed by removal of insoluble precipitates through adjusting the pH to 6.4 with 0.1 M–HCl and centrifugation[18]. The supernate after centrifugation was lyophilized. 4) Fingerprint maps were prepared by the method of Baglioni[19].

The abnormal peptide appeared on the map was eluted into 40% acetic acid, lyophilized after dividing the eluate into two tubes. The content of one of the tubes was hydrolysed with 6M–HCl at 105°C for amino acid analysis. Another tube was dealt with by partial hydrolysis with 5% acetic acid at 105°C for 8 hrs after confirming the existence of aspartic acid residues in abnormal peptide[20]. The partial hydrolysates was fingerprinted in the same way as in the analysis of tryptic peptide.

The amino acid analysis was performed in an automatic amino acid

analyser (YANACO, LC-7)

CASE REPORT

In 1977 a 28 yr-old housewife was admitted to the Sapporo Medical College Hospital in Sapporo, Hokkaido, for detailed hematologic observations. At age ten, she was noticed of jaundice and splenomegaly by her family physician. Since that time, she had continuously slight icterus, although she had no trouble in daily life. At twenty-six, she became pregnant and delivered an apparently normal infant.

In November 1977, she took cold. This induced aggravation of jaundice, enlargement of the spleen and dark discoloration of urine. Heinz body hemolytic anemia was suspected, because intraerythrocytic inclusion bodies were found in her peripheral blood and her hemolysate showed positive test for isopropanol precipitation.

Routine hematological and chemical examinations performed at that time were as follows: Hb 9.6 g/dl, PCV 0.32 1/1, RBC 3.29 x 10^{12}/1, MCV 9.7 fl, MCH 29.1 pg, MCHC 29.9 g/dl, WBC 4.9 x 10^{9}/1, Reticulocyte count 12.8%, Icteric Index 11, Total bilirubin 2.0 mg/dl, serum iron 108 γ/dl. The half life span of ^{59}Cr labelled erythrocyte was 5.2 day. There were a considerable number of erythrocytes with single Heinz body in peripheral blood.

Her parents and her four brethren were normal and their hemolysates were negative for isopropanol precipitation test. However, her daughter, her only child, was positive for the same test.

RESULTS

Cellulose acetate membrane electrophoresis of the freshly prepared hemolysate of the patient exhibited the discrete bands of Hb A_2, an abnormal hemoglobin ($\doteq$Hb X) and Hb A lining up in the order described from cathode to anode. The proportion of Hb X to total hemoglobin was 6.1 per cent. Isoelectric focusing of fresh hemolysate demonstrated the presence of several hemoglobin stripes of different migrations over the range from the Hb A band to the cathodic side beyond Hb A_2 band (Fig 1). These stripes increased in number when hemolysate was stored in a refregerator for several weeks.

The contents of Hb F and Hb A_2 were normal, being 1.2 and 2.8 per cent, respectively.

Heat instability test and isopropanol precipitation test of the hemolysate were positive. The purified abnormal hemoglobin solution showed an absorption spectrum of oxy-Hb type which was the same in shape as that of normal control Hb A solution over the visible region. However, its absorbance ratio E_{540}/E_{280} was 0.316, being distinctly smaller than the ratio in normal hemoglobin

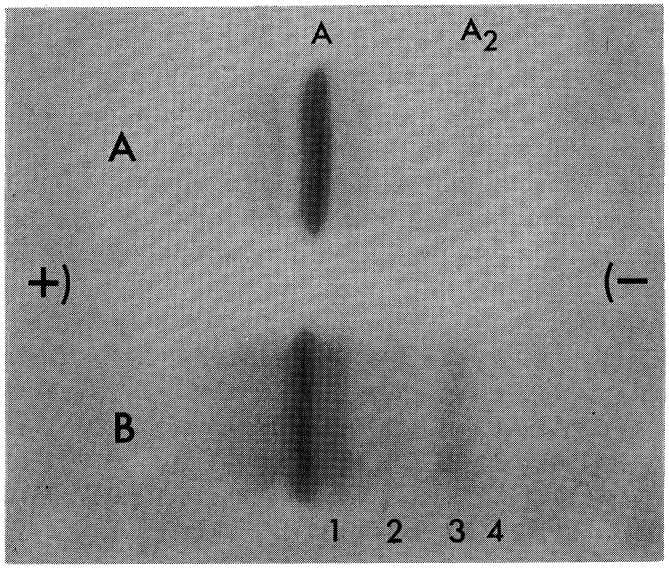


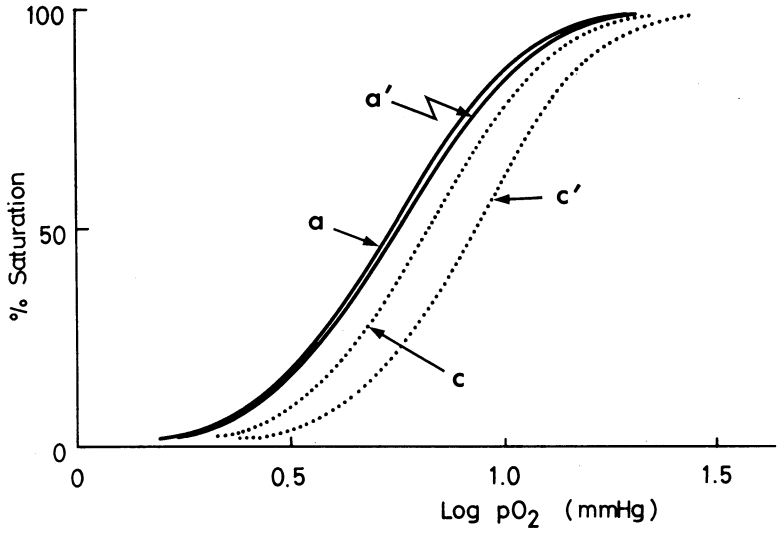
Fig. 1. Isoelectric focusing on polyacrylamide gel of the hemolysate of the patient. A: Normal subject, B: patient. 1,2,3 and 4 refer to stripes of the abnormal hemoglobin (Hb X). Stripe 3 comprises Hb A_2 in addition to Hb A_2 band.

(= 0.401). This indicates that in the abnormal hemoglobin there was loss of heme from its molecule by 21–24%.

Oxygen equilibrium curves of the abnormal hemoglobin revealed a left shift connoting high oxygen affinity, log $P_{50}=0.75$ at pH 7.0 (in Hb A, 0.93), and log $p_{50} = 0.73$ at pH 7.4 (in Hb A, 0.82). The heme-heme interaction was decreased slightly: Hill's n was 2.6−2.8 (in Hb A, 3.0). The alkaline Bohr effect was extremely small: $\Delta\log P_{50}/\Delta pH = -0.04$ (in Hb A, −0.28) (Fig. 2). The electrophoregram of the PCMB treated hemolysate disclosed the absence of β chain band, although the α chain band was visualized at the position pertaining to the normal α^A chain (Fig. 3). This was suggestive of a β-chain anomaly.

The fingerprint of the tryptic digest of aminoethylated abnormal β ($=\beta^X$) chain closely resembled that of the normal aminoethylated β chain. However, specific staining test and amino acid analysis of the eluates of the fingerprint spot revealed the presence of methionyl residue in β^XTp-11. This should have been negative if the β^XTp-11 had been identical with normal βTp-11. The result of the amino acid hydrolysis of the abnormal β^XTp-11 were as follows: His 0.86 (1), Arg 1.01 (1), Asp 2.21 (2), Glu 0.82 (1), Pro 0.64 (1), Val 0.15 (1), Met 0.91 (0), Leu 1.12 (1) and Phe 0.70 (1). The values in the parentheses

Fig. 2. Oxygen equilibrium curves of the purified hemoglobin solution.



Solid and dotted lines refer to Hb X and Hb A solution respectively. Lines a and c were measured at pH 7.4 and those of a' and c' at pH 7.0.

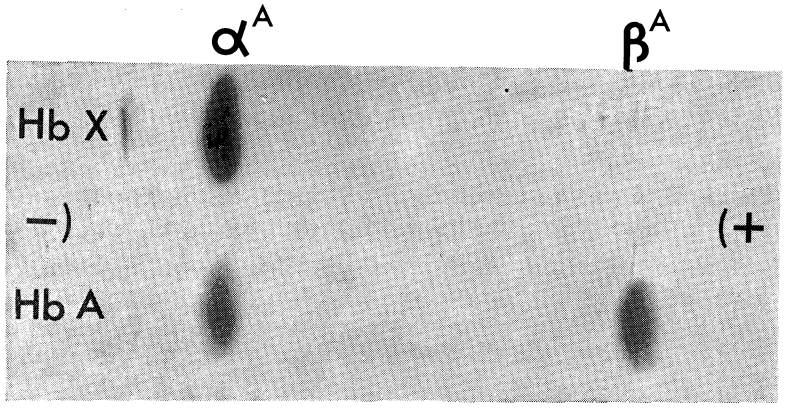


Fig. 3. Starch gel electrophoresis of the preliminarily PCMB treated hemolysate. Hb X: the patient, Hb A: normal subject.

refer to the numbers of residue present in the normal βTp-11 (Table 1). It is, therefore, inferred from this result that Val residue which occupies the 98th position of the normal β chain is substituted for by methionine in the

abnormal β^X chain. All the peptides except the β^XTp-11 was entirely the same in amino acid composition as the peptide fragments of the normal β^A chain.

For the purpose of confirming this amino acid substitution, the hydrolysate of the abnormal β^XTp-11 treated by partial hydrolysis with 5% acetic acid which would cleave the peptide at the site of Asp residue specifically was fingerprinted. Two spots, A and B were seen in the same pattern as in the control β^ATp-11 (Fig. 4). The amino acid analysis of the spot A revealed

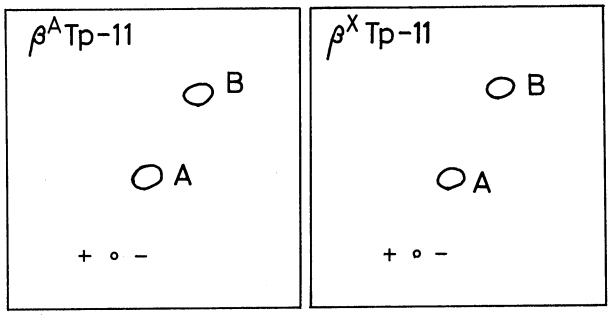


Fig. 4. Fingerprint of the acid hydrolysates of abnormal β^XTp-11 and normal β^ATp-11 with 5% acetic acid.

Arg 0.85 (1), Asp 1.02 (1), Glu 1.14 (1), Pro 0.86 (1) and Phe 0.70 (1). This corresponded to the residues occupying the 100th to the 104th sites of the normal β chain (Table 1). That of the spot B gave His 0.85 (1), Met 1.15

TABLE 1. Amino acid sequences of βTp-11 and β^XTp-11, and their partial hydrolysis with 5% acetic acid

	96	97	98	99	100	101	102	103	104
β^ATp-11	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg
β^XTp-11	Leu	His	Met	Asp	Pro	Glu	Asn	Phe	Arg
Partial hydrolysis	← spot B →				← spot A →				

(0), Leu 0.66 (1), and Val 0.0 (1). As witnessed from Table 1, this analytical result provides another evidence for substitution of Met for Val (98). The 99th Asp residue was not seen in the fingerprint map, probably because it was too fast in electrophoretic migration towards the anode to be arrested in the realm of the fingerprint map.

DISCUSSION

It is apparent from the result of structural analysis that the abnormal hemoglobin found in the blood of the patient is Hb Köln $\alpha_2{}^A\beta_2$ (98 Val→Met).

According to Perutz's hemoglobin molecule model[21], the residue FG-5 Val is at the 98th site in the β chain, and Val is common for FG-5 in all of the α, the β, the γ and the δ chain of human hemoglobins. Accordingly, the FG-5 Val residue is thought to play an important role in the structural stability and in the maintenance of function of hemoglobin molecule. Really, the residue participates in heme contact and in α_1-β_2 interphace interaction[22,23].

Therefore, the replacement of the FG-5 Val residue by other amino acid such as methionine, which is considerably larger in size than Val as in Hb Köln, by Gly in Hb Nottingham[24] or by Ala in Hb Djelfa[25], causes functional impairment or molecular instability. Detailed account of the cause of such defects has already been given in the text books[26]. Our patient was consistent with previously reported Hb Köln cases in physical and hematologic findings and in hemoglobin studies.

Hb Köln appeared to occupy only 6 per cent of total hemoglobin in our patient when examined by cellulose acetate membrane electrophoresis. This is unexpectedly low values as compared with the values of 10-15 per cent reported by other authors[27]. However, the Hb X stripes which were slower in anode-ward migration than Hb A in isoelectric focusing grew increasingly in number and intensity with the lapse of time during storage in a refrigerator for several weeks. This phenomenon will be due to increasing loss of heme from the abnormal hemoglobin fraction which progresses in parallel with the prolongation of storage period. Hb Köln will be the same as Hb A in electrophoretic migration shortly after it has been produced so long as its molecule has four heme as does Hb A. Therefore, the Hb A band comprises Hb Köln of intact molecule in addition to Hb A when fresh hemolysate is subjected to electrophoresis. By aging during storage of the hemolysate Hb Köln undergoes degeneration which is directly related to loss of heme from its molecule. The loss of heme results in decrement of negative electric charge of the Hb Köln molecule. Thus, Hb Köln becomes demonstrable as slow moving hemoglobin stripes by electrophoresis. The stripes which constitute abnormal hemoglobin fraction increases by aging of the hemolysate as evidenced by isoelectric focusing. The content of Hb Köln will therefore be estimated smaller when very fresh hemolysate is examined by electrophoresis than aged hemolysate is electrophoresed.

The fingerprint map of Hb Köln can not be discriminated from that of

HbA unless methionine spot staining is applied to the detection of individual tryptic peptides. This is an evidence for the supposition that the globin of Hb Köln is essentially the same as that of Hb A in electric charge.

Apparent absence of β chain demonstrable by starch gel electrophoresis of the PCMB-treated hemolysate will be accounted for by the degeneration of the β chain which has lost heme. The β^X chain which retains heme will be the same electrophoretic migration as the normal β^A chain, but it will vanish by PCMB treatment.

Hb Köln disease has been reported from three districts of Japan, namely Ube[8], Sendai[9] and Tokyo[10]. Accordingly, the present case is the fourth instance of Hb Köln disease in this country. This hemoglobinopathy is often the result of new mutation: the abnormal hemoglobin was not demonstrable in the blood samples collected from the parents of the propositi. However, it was detected in the blood of some of their children, either male or female. This is referred to the autosomal dominant inheritance of the gene of Hb Köln disease.

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