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AN α-THALASSEMIA FAMILY PRODUCING HEMOGLOBIN H AND ITS MODIFIED PIGMENT ELECTROPHORETICALLY INDISTINGUISHABLE FROM Hb BART'S

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Abstract

An α-Thalassemia family producing two electrophoretically fast-moving components of hemoglobins in addition to normal hemoglobins was encountered in our laboratories. The proband was a 23-year-old Thai female with microcytic hypochromic hemolytic anemia. The same abnormalities were seen also in her two sisters and in her father.

Of the two abnormal hemoglobins, the faster component (F-1) migrated to the position of ordinary Hb H, while another to that of Hb Bart's.

However, the identity of both of these pigments with Hb H was established by globin dissociation (into α and β chain) starch gel electrophoresis, fingeprinting and amino acid analyses. The differences in characters between the two pigments were as follows: 1) there was heme depletion, and the heme contents of F-1 and F-2 were 85 and 77 % of the theoretically expected, respectively. 2) titrative PCMB consumption was not equal (F-1=8.0, F-2=7.2, Hb A=2.0 mol PCMB/mol-Hb).

Tryptophan notch (289 nm) was not seen in both F-1 and F-2 just like in Hb A. Hemichrome absorption spectra instead of those of met Hb were seen in F-1 and F-2 in the process of oxidation. Oxygen affinity of these two pigments was increased over that of Hb A (F-1 and F-2, $P_{50} \fallingdotseq 0.3$; Hb A, 5.2-12.5 mmHg) with neither significant heme-heme interaction (Hill's n=1.0 in F-1 and F-2; n=2.6 in Hb A) nor the Bohr effect. From these findings it was presumed that F-1 was Hb H, and F-2 would be its modification generated by more marked heme depletion (23 %). The component F-2 should not be confused with Hb Bart's because they will behave electrophoretically in the same way.

INTRODUCTION

Hemoglobin (Hb) H disease is a hereditary microcytic hypochromic hemolytic anemia associated with production of Hb H (β_4) and Hb Bart's (γ_4)[1-4]. Etiologically it is conceived to be due to suppression of production of α chain, relative to its counterpart polypeptide chain, the β or the γ. The excess of β chain, in the adulthood, results the information of Hb H by single chain polymerization (β_4). This precipitates in the cytoplasm of red cells and leads them to hemolysis on account of damage to their cellular membrane.

Genetically, this disease is double heterozygous for α-Thalassemia genes, the αTh-1 and the αTh-2. The αTh-1 gene is of a severer type destined to produce no α chain, while the αTh-2 gene is of a milder type with decreased rate of α chain production.

When the Hb H disease patient's hemolysate is subjected to electrophoresis at pH 8.6, there revealed a characteristic pattern demonstrating various amounts of Hb H (β_4) and Hb Bart's (γ_4) as fast moving abnormal hemoglobins along with Hb A (the adult hemoglobin).

Recently, we happened to encounter a patient with this disease showing two extrapigments, one of which was Hb H and the other was like Hb Bart's but actually different from it.

The following are the report of our studies of these abnormal hemoglobins and some new contribution to the knowledge of this disease.

MATERIALS AND METHODS

Routine hematological and chemical tests were carried out by the method of standard procedures.[5]

Electrophoretic analyses (cellulose acetate, starch gel, agar gel, pH 8.6) were performed by the methods well-established by us in the past.[6-8] Purification and separation of desired hemoglobin components were processed by means of cellulose acetate electrophoresis (tris-EDTA-borate buffer, 0.05 M, pH 8.6) followed by cutting out individual stripes and their elution into tris-EDTA-borate buffer (0.05 M, pH 8.6) or phosphate buffer solutions (0.1 M, pH 7.0 and 6.5). Then the solution was centrifuged at 18,000 rpm below 4°C for 45 min to get purified components as clear supernatant.

Absorption spectra of the pigment were measured in a Cary type 118 automatic spectrophotomer.[9]

All the techniques necessary for structural analysis, including PCMB starch gel electrophoresis for identification of α and β chain, prepara-

tion of the globin, fractionation of α and β chain by means of 8M-Urea CMC chromatography, fingerprinting, amino acid analyses of the acid hydrolysate of the abnormal peptide were carried out, employing the procedures established in our laboratory.[6-8]

RESULTS

I) Case report: a 23-year-old female

The patient visited Japan as a scholarship student.

In April of 1975, she was detected to have microcytic hypochromic hemolytic anemia in the Tokyo University Hospital.

Hematological examination at that time revealed Hb, 8.4 g/dl; Ht, 31.3 %; RBC, 4.59×10^6 mm^3; MCV, 68 μm^3; MCH, 18.4 pg; MCHC, 27 %; reticulocyte count 12.8 %. Peripheral blood smear showed hypochromic microcytic anemia with occasional target cells and intraerythrocytic Heinz bodies in superavital staining with brilliant cresyl blue. No abnormalities were found in leukocyte count and its differential. Blood chemistry indicated total bilirubin, 1.6 mg %, (indirect bilirubin, 67 %); serum iron, 38 μg/dl; total iron binding capacity, 132 μg/dl, and no other chemical components were noticed to be abnormal.

Osmotic fragility was increased and acetyl phenylhydrazine Heinz body formation test was slightly positive.[8]

Intraerythrocytic enzyme assay of the Embden-Meyerhof pathway was not remarkable except for decrease in triose phosphate isomerase to about a half of the normal.[9] 2, 3-diphosphoglycerate concentration in the red cells was 2.25 mol/mol-Hb, indicating an incaease approximately as two-fold as high the normal (1.05 mol/mol-Hb).[9,10]

δ-Aminolevulinic acid (ALA) synthetase activity of the bone marrow was decreased remarkably to 2 nmol ALA/10^8 erythroblast-30 min as compared with the normal value of 23-38 nmol/10^8 erythroblast-30 min, and the inactivator of this enzyme was also decreased to about a half of the normal demonstrating dysfunction of heme synthesis.[11,12]

II) Hemoglobin study

Hemolysate was prepared in traditional way using H_2O and CCl_4, and subjected it to cellulose acetate membrane electrophoresis (pH 8.6).

Electrophoregram (pH 8.6) of the propositus demonstrated two extra fast-moving bands as compared with the normal control which showed Hb A_1 and Hb A_2 (Fig. 1). The pattern was similar in an elder and a younger sister as well as in father.

The stripes of the extra components were designated F-1 and F-2 in

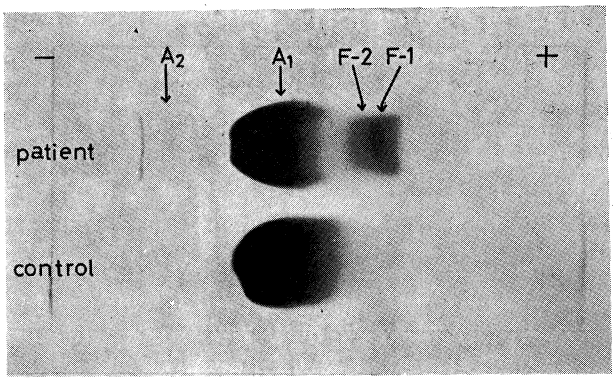

Fig. 1. Acetate membrane electrophoregram (pH 8.6) of the hemolysate of the propositus. F-1 and F-2 indicate fast-moving abnormal hemoglobin.

the order from the anode to the cathode, respectively (Hb A_1 and Hb A_2 were correspondingly F-3 and F-4).

The F-1 component separated in a sharp narrow band, while the F-2 component was less sharply delineated.

The contents in hemolysate of these components were F-1 : F-2 : F-3(A_1) : F-4(A_2)=9.7 : 4.7 : 84.1 : 1.5 %, respectively. The content of Hb F was 1.5 % by alkali denaturation test of the hemolysate, and no Hb Constant Spring was demonstrable even after benzidine or Ponceau staining of the electrophoresed cellulose acetate membrane.

Similar phenomena were also seen in agar gel and starch gel electrophoresis, but the discrimination of the two bands, the F_1 and the F_2, seemed to be inferior to that obtained by cellulose acetate membrane.

Heat denaturation by Carrel technique showed a slight increase (3.7 %) against the normal value (1.7-2.3 %).[13]

III) Functional studies

The visual absorption spectra of the components F-1, F-2 and F-3 (Hb A_1) were in good agreement with the spectrum of the normal control hemolysate.[14] Their Hüfners quotients were about 1.64. Soret peaks of these three component were within 414-414.5 nm (Fig. 2). However, the ultraviolet spectra showed higher absorbance in F-1 and F-2 than in normal control when the absorbance in visible region was adjusted to the same height (Fig. 3).

This finding was thought to be suggestive of heme depletion in F-1 and F-2 components. Accordingly, equimolar Hb solutions of F-1, F-2 and the normal control were prepared by adjusting the absorbance at

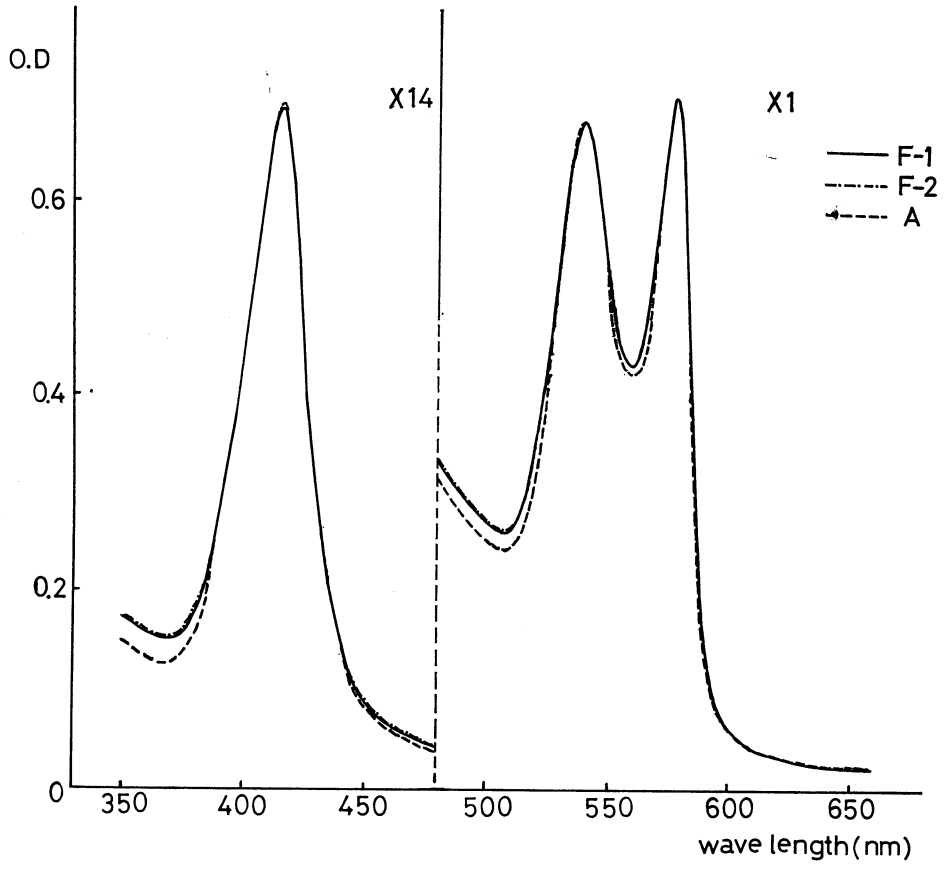

Fig. 2. Absorption spectrum (pH 7.0) of the purified abnormal hemoglobin in visible region.

wave length 275 nm to 0.985 (7.14 μM as Hb concentration) and their heme concentrations were determined by means of alkali hemochrome technique.

The results obtained in this experiment (Table 1) led us to presume that heme content of F-1 and F-2 corresponded to 85 and 77 % of the theoretically expected, respectively.

Both F-1 and F-2 showed no significant tryptophan notch (289 nm). This excluded their possibility of being Hb F and Hb Bart's possessing the γ chains.

The absorption spectra of acid met Hb type solution (pH 6.5) of F-1 and F-2 which were prepared by addition of $K_3Fe(CN)_6$ followed to

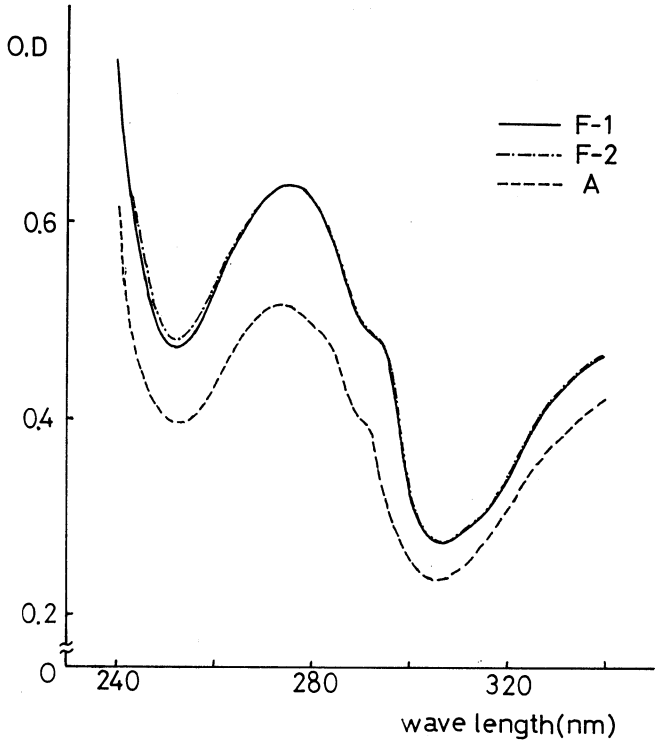

Fig. 3. Ultraviolet absorption spectrum (pH 7.0) of the purified abnormal hemoglobins after adjusting the pigments to the same absorbance at wave length 540 nm.

TABLE 1.

The heme content of the abnormal hemoglobins. E_{275} is the absorbance at wave length of 275 nm of the individual hemoglobin and E_{556} is the absorbance at wave length 566 nm of the alkaline hemochrome pigment. Alkaline hemochromes were prepared at follows: 2.0 ml hemoglobin solution + 10 mg $Na_2S_2O_4$ + 1.0 ml 10 % NaOH.

Sample	E_{275}	E_{556}	E_{556}/E_{275}	Heme Content %
Control A	0.973	0.500	0.514	100
F-1	0.984	0.434	0.441	85
F-2	0.986	0.393	0.399	77

dialize against 0.1 M-phosphate buffer to remove excess ferricyanide ion gave peculiar shapes which are depicted in Fig. 4.

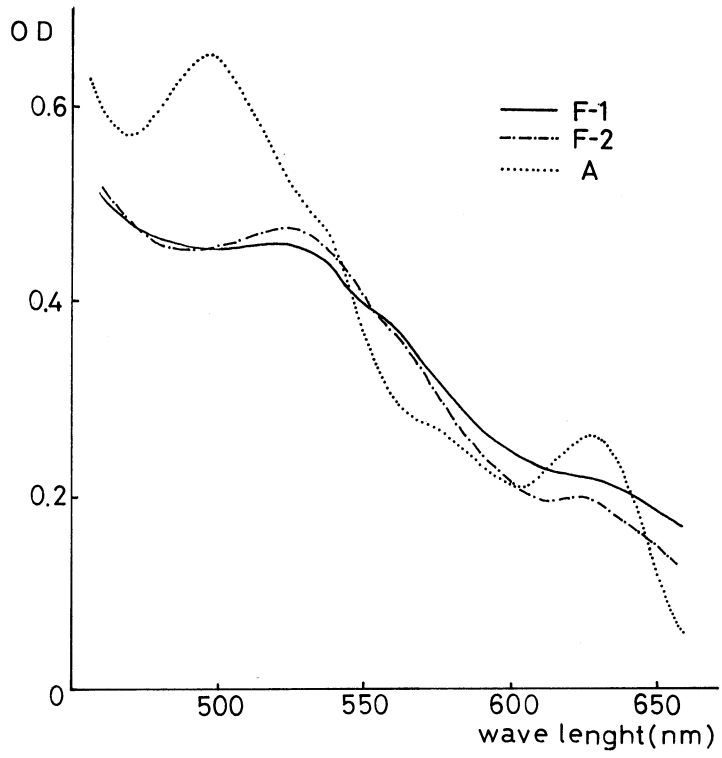

Fig. 4. Absorption spectrum of the acid methemoglobin type (pH 6.5) of abnormal hemoglobins. Notice to the hemichrome formation in the F-1 and the F-2 components.

Both F-3 (Hb A_1 fraction of the patient) and the normal control (Hb A_1) yielded typical spectra of acid met Hb A. In contrast, F-1 and F-2 were consistent with hemichrome, in the shape of absorption spectra, showing a bottom around 500 nm, a peak around 530 nm and no significant peak around 630 nm.[15]

Oxygen dissociation curves of F-1 and F-2 at various pHs (7.0, 7.4, 7.9) disclosed a marked left shift with $P_{50} \div 0.3$ mmHg, making a sharp contrast to $P_{50}=5.2$-12.5 mmHg of the normal adult hemoglobin. Heme-heme interaction was absent as indicated by Hill's $n=1.0$, and the alkaline Bohr effect was absent.[16]

IV) Structural studies

In the PCMB starch gel electrophoresis for identification of α and β chain at pH 8.6, F-1 was found to be consisted of β chain only, and F-2 to possess β chain as the major component with minimal amount of α

chain. The normal control revealed clearly delineated bands of the β chain, the undissociated $\alpha_2\beta_2$ (Hb A_1) and the α chain in the order from the anode to the cathode. Similar results were obtained on the 8 M Urea CMC chromatography (pH 8.6). These results refer the F-2 component to be different one from the F-1, although it is composed of almost pure β chain.

Fingerprinting of the trypsin digest of both F-1 and F-2 components showed that all the peptide spots seen on the map were identical

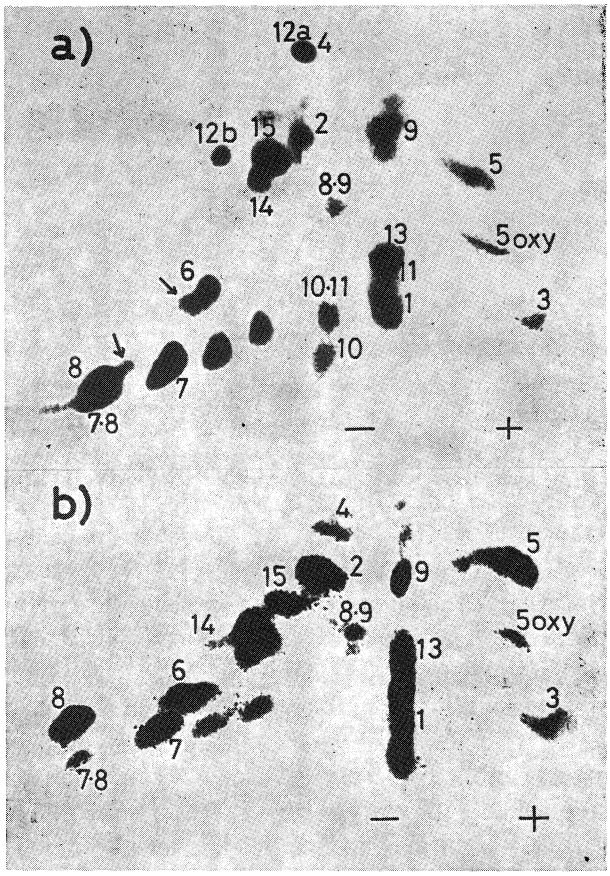

Fig. 5. Fingerprinting of the abnormal hemoglobin.
a) Fingerprint of aminoethylated globin of the F-1 component. Spot numbers are those of the β tryptic peptide chain. Two arrows show the contaminant of unknown origin after amino acid analysis. b) Fingerprint of the globin of the F-2 component. Spot numbers are those of the β tryptic peptide chain.

with those of β^A chain; and, in addition, no other spots which belong to or reminiscent of either γ^F, δ or α^A chain. However, in the fingerprint of F-1, there were two questionable extraspots which were finally concluded to be contaminants after examination of their amino acid compositions in comparison with the other ordinal spots on the map (Fig. 5).

DISCUSSION

Fast moving F-1 and F-2 components which were clearly discriminated from each other by cellulose acetate membrane electrophoresis were proved to be solely composed of β chain by PCMB starch gel electrophoresis and the fingerprinting. The supposition that both pigments to be Hb H(β_4) and its modification is supported by absence of tryptophan notch (289 nm) in their absorption spectra and their titrative PCMB consumption values (8.0 (F-1) and 7.1 (F-2) mol PCMB/mol-Hb) which will be published in detail elsewhere.

Accordingly, the propositus who presented hypochromic microcytic hemolytic anemia would be diagnosed as a patient with Hb H disease, genetically double heterozygous for α-Thalassemia-1 and α-Thalassemia-2 genes. The results of hematological and clinical chemistry examinations are also consistent with this disease.

It is worthy of special mentioning that there was seen an electrophoretic segregation of Hb H into two components in this patient. The F-2, which came electrophoretically between the ordinary Hb H and Hb A, might have been traditionally regarded as Hb Bart's by routine screening of hemolobinopathies. However, it was truly a modified Hb H in the family of our patient. Therefore, a careful examination will be necessary when the F-2-like electrophoretic stripe is encountered.

Appearance of these two Hb H, the F-1 and the F-2, might be elucidated by the difference in the degree of heme loss. The F-1 and the F-2 lost heme in 15 and 23 per cent of the theoretically expected values, respectively.

It was already reported by Winterhalter that in Hb A heme loss Hb A results in slower electrophoretic migration variously depending on the level of heme depletion, and, in addition instabilities of molecule.[17,18] This fact might be expected to be analogously seen in the case of Hb H disease. However, the performance of direct experimental verification was impossible for us with Hb H disease, because the F-2 component and Hb H were so unstable.

The cause of heme depletion in the F-1 and the F-2 is unclear.

Whether it might arise from detachment of heme from fully heme-combined hemoglobin molecule on account of its instability or by decreased heme supply because of diminution of ALA synthetase activity is still open to question.

2, 3-diphosphoglycerate in the patient's red cells was increased about two times the normal level. This increase may not be explained simply by the tendency toward increased regeneration of younger red cell (red cells rich in enzymes), since almost all of the intraerythrocytic Embden-Meyerhof pathway enzymes except triose phosphate isomerase were within the normal range. It may therefore be accounted for by the lowered oxygen affinity of Hb H.

It was already reported by various authors that the concentration of 2, 3-diphosphoglycerate had no relationship to the hemoglobinopathies producing abnormal hemoglobins of high oxygen affinity.[19,20] Therefore, the increase of 2, 3-PDG in our patient may suggest the possibility that α-Thalassemia will etiologically be somewhat different from the ordinary hemoglobinopathy which creates abnormal hemoglobin by one-point mutation or deletion of the hemoglobin genes. In this respect it resembles hereditary enzyme deficient hemolytic anemias which show high O_2 affinity of hemoglobin and increased 2, 3-DPG.

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