Hemoglobin Hikari ($\alpha_2^A\beta_2^{61AspNH_2}$): A New Abnormal Hemoglobin Detected from Two Unrelated Japanese Families

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Until recently there was a consensus of opinion among the hematologists that hemoglobinopathy was not distributed in Japan. This conception which was not based on concrete observation was soon overthrown.[1] A series of systematic survey conducted in Tokyo, Kyoto, Ube and Fukuoka has been demonstrating the presence of a variety of hemoglobinopathy in this country since 1960.[2] As many as thirteen abnormal hemoglobins have been recorded to date.[3]

The present paper is concerned with an abnormal hemoglobin discovered electrophoretically from a man living in Hikari, Yamaguchi Prefecture, in our laboratory in 1961.[4] He was 22 year old and had neurasthenia. This abnormal hemoglobin, which did not produce symptoms, migrated toward the anode ahead of Hb A but a little behind Hb N on electrophoresis (pH 8.6). This hemoglobin was designated Hb Hikari after the name of the town where the propositus lived.

About three months after the discovery of this hemoglobin, another one resembling closely in electrophoretic migration was detected from a cyanotic 6 year old boy dwelling in Ube, Yamaguchi Prefecture. This second hemoglobin was named Hb Ube 3 tentatively.

A comparative study of the two hemoglobins including hybridization, urea dissociation paper electrophoresis, fingerprinting and amino acid analysis of the extract of the abnormal peptide has lately established their complete identity.

Genetic study disclosed the absence of marital relation between the families of Hikari and Ube. However, it deserved special mention that the mothers of both propositi were the carriers who introduced the abnormal hemoglobin into their families and that they were alike the descendants of Yonago district, Tottori Prefecture.

Inasmuch as the incidence of hemoglobinopathy is considerably low in this country,[1,2,3] it is thought to be quite an unusual event that a particular same hemoglobin is found encompassing two independent families. In this connection, Hb Hikari is to be ranked among the most notable of the Japanese abnormal hemoglobins.

This paper aims to present our chemical study on Hb Hikari and Hb Ube 3.

CASE RECORD

The first family: —T. A., a 22 year old male was the propositus. His mother was born in Yonago district, Tottori Prefecture. He was working in a mercuric insecticide factory in Hikari, Yamaguchi Prefecture. The patient has been complaining of fatiguability, loss of appetite, inosomnia, slight cough and rigidness of the shoulders since July 1961. He was told to have an old pleurisy by X ray examination of the chest. He was seen in the outpatient clinic of the Yamaguchi Medical College Hospital with a chief complaint of easy irritability.

His past and family histories were not remarkable and physical examination was negative.

Laboratory examinations disclosed the following: —

Examination of the blood, which was repeated on three occasions from August 1961 to the present time, was within normal limits.

Blood chemistry, also repeated three times, revealed that hemoglobin was 16.3g/dl; serum protein, 7.6–7.8g/dl; A/G, 1.33–1.60; albumin, 4.4–4.8g/dl; globulin, 3.0–3.3g/dl; blood sugar, 60–94g/dl; icteric index, 4–5; CCFT, 0—2+; cholinesterase, 1.1—0.9ΔpH; alkaline phosphatase, 1.9–2.3 Bodansky units; cholesterol, 174–235 mg/dl; phenol turbidity test, 14–21 Kunkel units; non protein nitrogen, 16–21 mg/dl; urea N, 7.5–9mg/dl; and transaminase (GPT), 9.0–34 Mizobe units. These findings were consistent with slight hepatic diturbance.

Hemorrhagic studies: Bleeding time, coagulation time, clot retraction, prothrombin time and serum prothrombin time were within normal range.

Hemolytic studies: Cartwright's screening test[5] demonstrated that a normal osmotic fragility, a negative hemolysin (warm), a negative agglutinin (warm and cold) and positive hemolysin (cold). Coombs' test (direct and indirect)[6] and sickling test[7] were negative.

The urine was negative for protein, sugar and urobilinogen.

The second family: —The propositus was S.T., a 6 year old boy. His mother came from Yonago City, Tottori Prefecture. He was noticed to have cyanosis of the lip since his birth. He entered the Pediatric Department of the Yamaguchi Medical College Hospital in November, 1961 because of a polycythemia which happened to have been detected on the medical examination for entering the primary school.

Physical examination: Oral membrane and gingiva were dark red in color. There were clubbing and cyanosis of the fingers. The heart and lungs were normal to auscultation and percussion. The liver was palpable 1 finger-breadth below the right costal margin. However, the spleen and kidneys were not felt.

Examination of the blood: The red cell count was $803 \times 10^4/mm^3$; and the hematocrit, 63.0%. The platlet count, white cell count and differential count were

normal.

Blood chemistry: Hemoglobin was 18.3g/dl; serum protein, 6.4mg/dl; A/G, 0.95; albumin, 3.6g/dl; globulin, 3.8g/dl; blood sugar, 66mg/dl; icteric index, 6–7; CCFT, 1+; cholinesterase, 0.9 ΔpH; alkaline phosphatase, 2.7 Bodansky units; cholesterol, 110mg/dl; phenol turbidity test, 6 Kunkel units; non protein nitrogen, 25mg/dl; urea N, 12mg/dl; and transaminase (GPT), 6.5 Mizobe units.

Hemorrhagic studies: Clot retraction was normal and falling out of the blood corpuscle was not seen.

Hemolytic studies[5]: Osmotic fragility was normal, hemolysin (warm and cold) and agglutinin (warm and cold) were negative. Coombs' test[6] (direct and indirect) and sickling test[7] were also negative.

MATERIALS AND METHODS

1) Preparation of hemolysate: Blood was collected from the propositus (T.A.), his parents and sisters (No. 1 and No. 2) of the first family and the propositus (S.T.) and his mother of the second family. Hemolysates were prepared by the conventional method[8]: erythrocytes were washed and centrifuged three times with physiologic saline followed by the addition of a volume of distilled water two times as much as that of packed red cells and 0.5 volume of toluene. After the mixture was vigorously shaken to be hemolyzed the stroma was separated by centrifugation. The hemolysate contained about 10g/dl of hemoglobin.

2) The alkali denaturation test was examined by the method of Singer,[9] and the solubility of the reduced hemoglobin was studied by the method described by Goldberg.[10]

3) Electrophoretic studies: As for the agar gel electrophoresis Shibata and Iuchi[11] were followed and for the starch block electrophoresis Ueda's method[12] which used tris-EDTA-borate buffer solution of pH 8.6 and pH 7.0 was employed.

The content of the abnormal hemoglobin was measured by scanning the pattern of agar gel electrophoregram with a pholoelectric apparatus.

Paper electrophoresis was carried out by a modified Durrum-Flynn's method[13] using veronal buffer solution of pH 8.6 and phosphate buffer solution of pH 6.5.[14]

4) Amberlite IRC 50 chromatographic study was done by the method described by Huisman and Prins.[15]

5) Spectroscopy (650−380 mμ) of the abnormal hemoglobin (O_2 Hb type, pH 8.6; met Hb type, pH 7.0) which was purified by starch block electrophoresis was performed in a Shimazu QB spectrophotometer.

6) Chemical studies: The abnormal hemoglobins (Hb Hikari and Hb Ube 3) were isolated and purified by starch block electrophoresis. Polypeptide chain abnormality of thus purified hemoglobins were studied by urea dissociation paper electrophoresis (Take),[16] and by their hybridization with canine hemoglobin (Shibata

et al).[17] Then, their globin and their α and β chains, were prepared by Anson-Mirsky's method[18] and Hayashi's method,[19] respectively, in order to make their fingerprints by the methods of Ingram[2] and of Baglioni.[21] The abnormal spots found on the fingerprints were eluted and hydrolyzed with 6N HCl.[22] They were also eluted with 0.01 N HCl and neutralized with the addition of powdered sodium bicarbonate to be subjected to the hydrolysis with pronase.[23] The amino acid constituent of the hydrolysates was studied by a two-dimensional electrophoresis-chromatography technique.[20]

RERULTS

All the hemoglobin studies so far as performed in our laboratory were unanimously suggestive of the identity of the hemoglobin (Hb Hikari) found in the first family with that (Hb Ube 3) of the second family. Not a single significant difference in chemical properties was demonstrable between these hemoglonins.

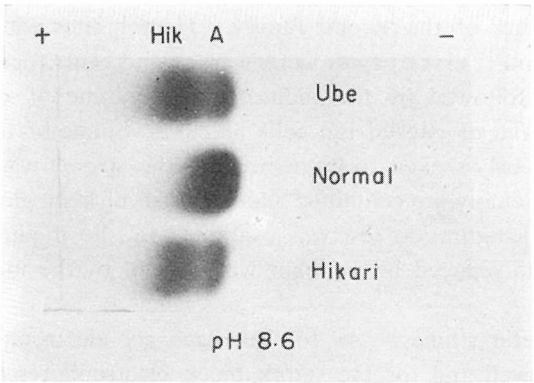


Figure 1. Agar gel electrophoresis of the hemolysates.
A : Hb A. Hik : Hb Hikari. Hikari : Propositus of the Hikari family.
Ube : Propositus of the Ube family. Normal : Normal person.

1) Agar gel electrophoresis (Figure 1): The hemolysates showed three distinct hemoglobin components, one with the mobility of hemoglobin A_1 and a second one of faster anodal mobility and a third one of the slowest mobility (Hb A_2) at pH 8.6, while at pH 7.0 no separation was obtained. Control experiment disclosed that the abnormal component is slightly slower in migration than Hb N at pH 8.6. The content of abnormal hemoglobin in the hemolysates was as follows: 60.8 per cent (propositus), 53.6 per cent (mother), and 69.1 per cent (sister) in the first family (Hb Hikari), and 66.1 per cent (propositus) in the second family (Hb Ube 3).

2) Paper electrophoresis (Figure 2): The abnormal hemoglobin moved faster than Hb A toward the anode at pH 8.6. No abnormal component was separable at pH 6.5.

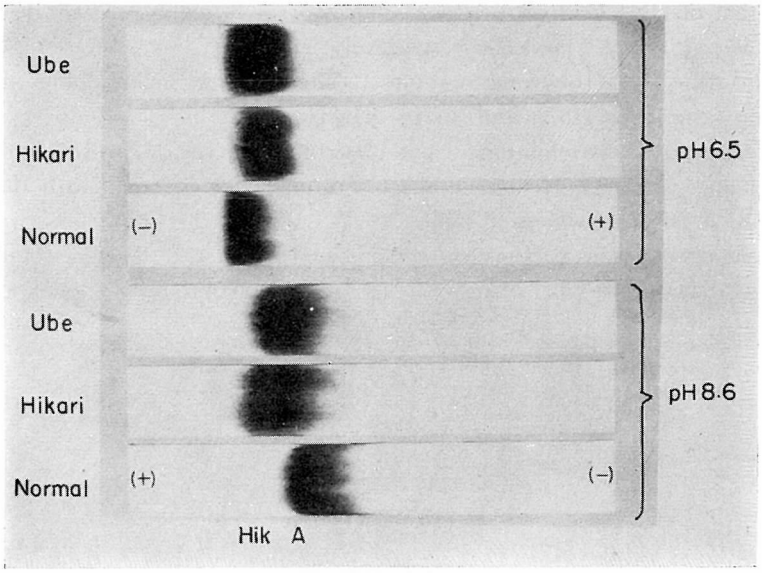


Figure 2. Paper electrophoresis of the hemolysates.
A : Hb A. Hik : Hb Hikari. Hikari : Propositus of the Hikari family. Ube : Propositus of the Ube family. Normal : Normal person.

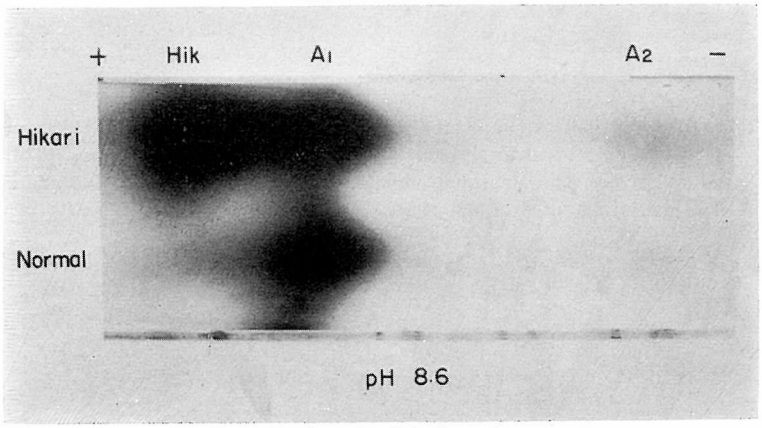


Figure 3. Starch block electrophoresis of the hemolysates.
A_1 : Hb A_1. A_2 : Hb A_2. Hik : Hb Hikari. Hikari : Propositus of the Hikari family. Normal : Normal person.

3) Starch block electrophoresis (Figure 3): The abnormal hemoglobin had a higher anodal mobility than Hb A_3 (minor component) and Hb A_1 at pH 8.6 and pH 7.0. Hb Hikari and Hb Ube 3 exhibited an identical electrophoretic migration on starch block as well as on agar and paper.

4) Solubility test and alkali denaturation test: The solubility of reduced hemoglobin was 96.5 per cent for the hemolysate of Hb Hikari propositus, and 100 per

cent for that of Hb Ube 3 propositus. The content of alkali-resistant hemoglobin was 1.8 per cent and 2.7 per cent, respectively.

5) Amberlite IRC 50 chromatography: The abnormal hemoglobins descended the column faster than Hb A and slower than Hb F.

6) Spectroscopic examination: The absorption curves of spectrography of the patients' hemolysates (O_2 Hb type and met Hb type) are identical with that of Hb A over the whole range of visual light.

7) Urea dissociation paper electrophoresis (Figure 4): The β chain of Hb Hikari and that of Hb Ube 3 showed the same electrophoretic anodal migration,

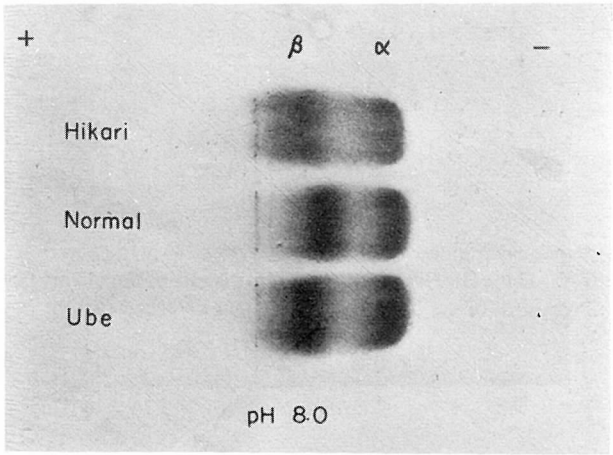


Figure 4. Urea dissociation paper electrophoresis of the globin of Hb Hikari. α : α chain. β : β chain. Hikari : The abnormal hemoglobin of the Hikari family. Ube : The abnormal hemoglobin of the Ube family. Normal : The hemoglobin of a normal person.

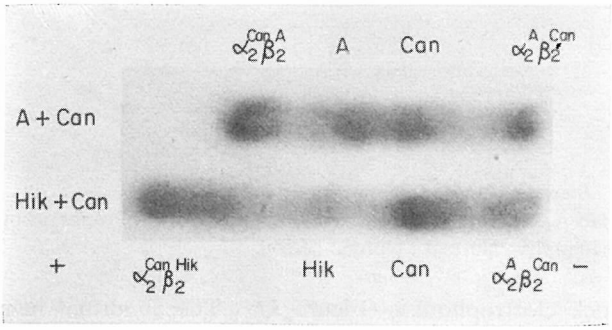


Figure 5. Hybridization test with canine hemoglobin.
A+Can : Hybridization with Hb A and Hb Can. Hik+Can : Hybridization with Hb Hikari and Hb Can. A : Hb A. Can : Hb Can. $\alpha_2^{Can}\beta_2^{A}$ and $\alpha_2^{Can}\beta_2^{Hik}$: Hybrids produced by the α chain of Hb Can and the β chain of Hb A or of Hb Hikari. $\alpha_2^{A}\beta_2^{Can}$: Hybrid produced by the α chain of Hb A and the β chain of Hb Can.

both being faster than the β chain of Hb A. The α chains could not be discriminated from the α^{A} chain.

8) Hybridization (Figure 5): There was distinct difference in the electrophoretic mobility between the hybrids appearing to the anode side, which were concerned with the hemoglobins composed of canine α chain and human β chain. Hybrid $\alpha_2^{Can}\beta_2^{Hikari}$ (and $\alpha_2^{Can}\beta_2^{Ube3}$) was faster than hybrid $\alpha_2^{Can}\beta_2^{A}$. Such a dyscrepancy was not demonstrable on the cathode side. $\alpha_2^{Hikari}\beta_2^{Can}$ (and $\alpha_2^{Ube3}\beta_2^{Can}$) and $\alpha_2^{A}\beta_2^{Can}$ were the same in electrophoretic mobility.

9) Fingerprinting (Figure. 6): Hb Hikari and Hb Ube 3 (whole globins) showed an abnormal fingerprint. Both of them had an abnormal spot which was clearly visible to the anode side of peptide spot #20, missing #19 peptide spot. Separatly

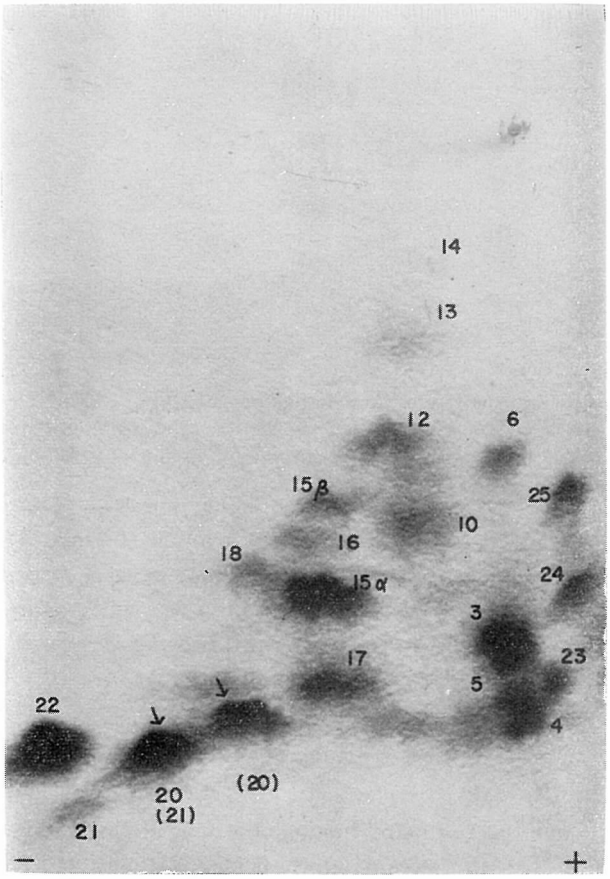


Figure 6. Baglioni's fingerprint of the whole globin of Hb Hikari.
Arrows indicate the abnormal peptides. Peptide 20 (21) is the mixture of peptide #20 (normal α chain) and the abnormal peptide (21) of the β chain of Hb Hikari which has Lys in addition to all the amino acids of the abnormal peptide (20). Peptide (20) refers to the abnormal peptide of the β chain of Hb Hikari.

purified α and β chains revealed that the abnormality was concerned with β chain contrasting entirely normal α chain.

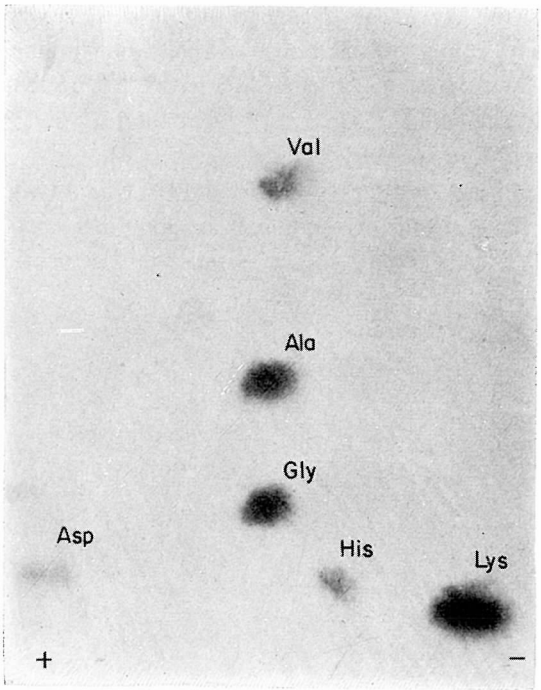


Figure 7. Amino acid analysis of the abnormal peptide (20).
The acid hydrolysate of the abnormal peptide was subjected to high potential paper electrophoresis horizontally in direction (+) − (−), and then developed by paper chromatography vertically.

10) Amino acid analysis of the abnormal peptide (Figure. 7): Acid (HCl) hyrolysis of the eluate of the abnormal spot adjascent to ♯20 peptide disclosed a hexapeptide which was composed of Lys, His, Gly, Ala, and Asp in both Hb Hikari and Hb Ube 3. However, hydrolysis with pronase demonstrated the presence of Asp NH_2 in addition to Lys, His, Gly, Ala, and Val.

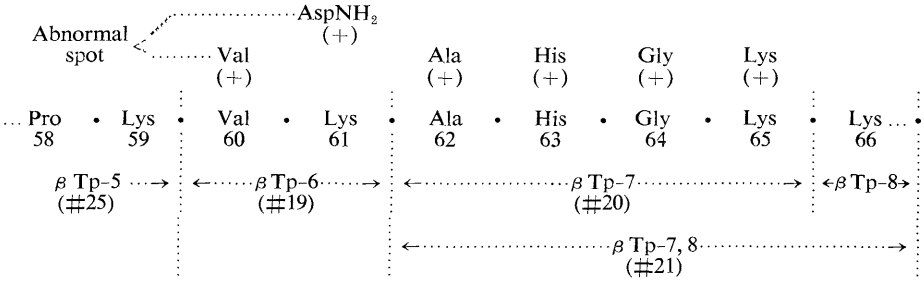
DISCUSSION

It is apparent from the results of hemoglobin study which have been described above that Hb Ube 3 of the second family is identical with Hb Hikari of the first family. They are exactly alike in electrophoreses (fast-moving on agar, paper and starch block), urea dissociation paper electrophoresis, hybridization test (β chain anomaly), fingerpriting (disappearance of ♯19 peptide, β Tp-6, and an abnormal spot in the anodal adjacency of peptide ♯20) and amino acid analysis. There is

not a single evidence against the identidy of these hemoglobins.

Amino acid analysis of the abnormal spots eluted from the fingerprints of the β chains of Hb Hikari and Hb Ube 3 was interesting. The abnormal spot showed the presence of Asp NH_2 and Val in addition to all the amino acids belonging to the normal #20 peptide on hydrolysis with pronase, while Asp was demonstrated instead of Asp NH_2 on hydrolysis with HCl. It is therefore presumed that Asp NH_2 proper to the abnormal peptide was converted into Asp because of the release of NH_2 by the treatment with HCl. Comparison of the composition of the abnormal peptide with the established amino acid sequence of normal β chain, as presented in Table 1, discloses a close resemblance of the peptide to the union of peptide #19 with peptide #20.

Table 1. Amino acid composition of the peptide of the abnormal spot on fingerprint in comparison with the amino acid sequence of β Tp-6 (#19) and β Tp-7 (#20).



If the Lys occupying the 61st place of the normal β chain is substituted for by Asp NH_2, a peptide Val • Asp NH_2 • Ala • His • Gly • Lys which is the same in composition as the abnormal peptide will be produced by tryptic digestion of such a β chain, because trypsin cannot split the fusion of peptides #19 and #20 at the position of Asp NH_2 (β 61).

The absence of the spot of peptide #19 from the fingerprint of the β chains of Hb Hikari and Hb Ube 3 is accounted for as a natural consequence of this kind of amino acid substitution.

Asp NH_2 is less basic and more negatively charged than Lys. Substitution of Lys by Asp NH_2 in the β chain will result in an increase in negative charge of the relevant hemoglobin. This is consistent with the fast-moving electrophoretic behavior of Hb Hikari and Hb Ube 3. It is therefore concluded that Hb Hikari and Hb Ube 3 are the same and they are a hemoglobin expressed by $\alpha_2^A\beta_2^{61AspNH_2}$.

The pedigrees of the first and the second families are illustrated in Figure 8. It is worthy of special mention that the abnormal hemoglobins were introduced into both families by the mothers of the propositi, who were the natives of Yonago district, Tottori Prefecture. It is an unusual event in Japan that an abnormal hemoglobin of the same kind is discovered from two independent families without marital relation, because hemoglobinopathy is a rarity in this country. Hb Kokura

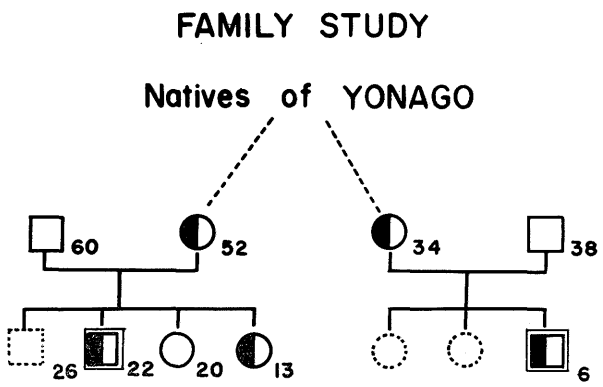


Figure 8. Pedigrees of Hb Hikari-hemoglobinopathy
Left : The Hikari family. Right : The Ube family.
Circles and squares with black and white areas indicate the heterozygotes of Hb A and Hb Hikari. Those without black area are normal persons, and those drawn in broken line are persons whose blood could not be examined.
Figures represent the ages. Squares encircled with double solid lines refers to the propositi.

is the only one additional example among the abnormal hemoglobins of Japan numbering more than ten.

Up to the present time three kinds of fast-moving hemoglobins have been recorded in Japan. They are Hb Ube 2, Hb Tokuchi and Hb Tsukiji.

Hb Ube 2 was discovered from a 44 year old woman with uterine cancer and her daughter living in Ube.[2] This hemoglobin is hardly distinguishable from Hb Hikari electrophoretically, but it is a hemoglobin of α chain anomaly which is represented by the fromula $\alpha_2^{\mathrm{Tp}-9}\beta_2^{\mathrm{A}}$.

Hb Tokuchi[2,3] moves slower than Hb A on agar gel electrophoresis at pH 7.0, and it is a hemoglobin possessing β chain anomaly which is expressed by the formula $\alpha_2^{\mathrm{A}}\beta_2^{2\mathrm{Tyr}}$.

Hb Tsukiji was discovered from a 28 year old pregnant woman in the St. Luke Hospital, Tsukiji, Tokyo.[24] This hemoglobin is slightly slower than Hb Hikari on agar gel electrophoresis (pH 8.6) and has β chain anomaly which is represented by the formula $\alpha_2^{\mathrm{A}}\beta_2^{\mathrm{Tp}-3}$.

It is accordingly apparent that Hb Hikari is different from these hemoglobins. Hb Hikari is a new hemoglobin found in Japan which has never been reported in the world.

CONCLUSION

A new fast moving abnormal hemoglobin (Hb Hikari) was discovered from two independent Japanese families without marital relation. This hemoglobin was not

associated with any clinical and hematological manifestions. Electrophoretically (agar, paper and starch block) it migrated between Hb A and Hb N at pH 8.6, but did not separate from Hb A at pH 7.0.

It produced a layer intermediate between Hb F and Hb A on Amberlite IRC 50 column chromatography.

The reduced hemoglobin solubility test and alkali denaturation test of Singer were normal.

The hemolysates contained the abnormal hemoglobin (about 60%) more abundantly than Hb A.

Hybridization test and urea dissociation paper electrophoresis disclosed its β chain anomaly.

The fingerprints of its whole globin and β chain presented a characteristic pattern: There was no spot relevant to peptide #19, while a distinctly abnormal spot was visible in anode-ward adjacency of peptide #20. Amino acid analysis of the abnormal spot demonstrated the presence of lysine, histidine, glycine, alanine, valine and asparagine. It was accordingly apparent that an abnormal peptide, which was an union of peptides #19 and #20, was formed by fusion through the substitution of the lysine residue occupying the 61st place of β chain by asparagine.

The hemoglobin had a chemical formaula expressed by $\alpha_2^A\beta_2^{61AspNH_2}$, and was designated Hb Hikari after the name of the town where the propositus of the first family lived.

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