Kawasaki Med. J. Vol. 2: No. 4, 185-197, 1976

THE THIRD INSTANCE OF Hb M SASKATOON DISEASE DISCOVERED IN JAPAN

Satoshi UEDA,* Susumu SHIBATA,* Yoshihito YAWATA,* Osamu YAMADA,* Takeshi NAGAO,** Takaoki MIYAJI*** and Yuzo OHBA***

*Department of Medicine, Kawasaki Medical School, Kurashiki, **Department of Hematology, Kanagawa Children's Medical Center, Yokohama, and ***Department of Clinical Pathology, Yamaguchi University School of Medicine, Ube.

Accepted for Publication on July 21, 1976

Abstract

A four-year-old girl who had been persistently cyanotic since her birth notwithstanding the absence of congenital heart disease was seen at the Kanagawa Children's Medical Center (Yokohama) in 1974.

The absorption spectrum of acid methemoglobin type hemolysate of this patient failed to show the peak at 630 nm and the depression at 600 nm which were characteristic of methemoglobin of a normal subject.

Cellulose acetate membrane electrophoresis (pH 7.1) of methemoglobin type hemolysate yielded a discrete greenish stripe of an abnormal hemoglobin to the anode side of the stripe of normal methemoglobin which was reddish brown.

The abnormal hemoglobin was purified and collected from the methemoglobin type hemolysate by cellulose acetate membrane electrophoresis (pH 7.1).

Exact investigation of the absorption curves of the acid met Hb type hemolysate and the purified acid met Hb type abnormal hemoglobin solution not only over the range of visual spectrum but also over the ultraviolet region, yielded the results reminiscent of Hb M Saskatoon.

The α and the β chains of the abnormal globin were aminoethylated, digested with trypsin, and fingerprinted. Amino acid analysis of abnormal peptide spots on the fingerprint revealed a β chain anomaly which was entirely consistent with Hb M Kurume ($\alpha_2\beta_2 63$ His $\rightarrow$ Tyr). This is the third instance of Hb M Saskatoon (M Kurume) discovered in Japan.

* This investigation was supported by the Grant from the Ministry of Health and Welfare of Japan for the Researches of Specified Diseases (Hemolytic Anemia) and for the Researches of Congenital Metabolic Abnormalities.

INTRODUCTION

Hb M ($\alpha_2^M\beta_2$, $\alpha_2\beta_2^M$) is a chocolate-colored hemoglobin with an abnormal α or β chain (α^M, β^M) of which proximal histidine (α87, β92) or distal histidine (α58, β63) is substituted for by tyrosine[1]. In the anomalous chain tyrosine, the substitute for histidine residue, binds to the oxidized heme iron ($Fe^{++}\rightarrow Fe^{+++}$) to stabilize the latter in methemoglobin form (heme iron in Fe^{+++}) which is a dark brown polypeptide incapable of reversible oxygen transportation.

The stability of hemoglobin molecule depends on β chain, and if the β chain is anomalous, the relevant hemoglobin molecule often becomes fragile on account of difficulty in maintaining its three-dimensional structure. Hb M's can neither be exceptions. In fact, Hb M Saskatoon (β63 histidine $\rightarrow$ tyrosine)[2] and Hb M Hyde Park[3] ($\equiv$ Hb M Akita[4], β92 histidine$\rightarrow$tyrosine), are unstable. They undergo degeneration even while they are still contained in erythrocytes, and impair their cellular membrane, thus rendering them into effete cells which are easily caught and destroyed by the splenic reticuloendothelial system before the expiry of their life span. Hemolytic anemia may, accordingly, ensue.

A patient with such an abnormal hemoglobin was discovered by Nagao at the Kanagawa Children's Medical Center in October, 1974. The hemoglobin of this patient was studied by the Department of Medicine, Kawasaki Medical School, Kurashiki, in collaboration with the Department of Clinical Pathology, Yamaguchi University School of Medicine, Ube, and was successfully identified as Hb M Saskatoon. The finding obtained in this study are presented hereunder.

CASE RECORDS

The patient was a four-year-old well developed girl with a pretty large stature. Her parents (the father being 33 years old, and the mother, 34) were normal. The child was born in normal labor at full term, weighed 2650 g and measured 48 cm at birth. Neonatal jaundice was moderate. Cyanosis was noticed in the face shortly after birth, but because the baby appeared normal, she was brought up without any particular medical care. However, the patient was warned of possessing systolic murmurs in the precordial region at six months after birth, and was suspected of having chronic heart disease at age three.

Physical examination:— Despite the violet discoloration over the whole body (skin and mucous membrane), the patient was in favorable general condition, and the cyanosis was not aggravated even after ex-

ercise. Splenomegaly and hepatomegaly were absent. The patient was normal except for cyanosis and positive urobilinogenuria. Blood O_2 content (vol. %) determined by cardiac catheterization was 3.86 in the right ventricle, 6.14 in the right auricle, and 7.65 in the left auricle (by inhalation of O_2), and the blood O_2 combining capacity was 15.4 vol. %.

Laboratory examination:— Blood hemoglobin was 11.6 g/dl; hematocrit 36 %; RBC 371×10^4 per cubic mm. Microscopy of the peripheral blood smear disclosed no particular abnormalities but slight anisocytosis. WBC count was 7400 cells per cubic mm, and leukocyte differential count was normal; platelet count was 16.8×10^4 cells per cubic mm. Reticulocyte count was 0.8~2.1 %. Serum haptoglobin was low (10 mg/dl).

Serum protein was 7.3 g/dl; serum bilirubin, 0.6 mg/dl; BUN, 13 mg/dl; serum sodium, 139 mEq/l; serum potassium, 4.0 mEq/l; serum chloride, 106 mEq/l: plasma fibrinogen, 210 mg/dl; plasma prothrombin time, 12.5″; partial thromboplastin time, normal (45″).

Because of the presence of cyanosis, the blood was assayed for methemoglobin (by Evelyn-Malloy's method), and its content was proved to be not more than 0.3 %.

METHODS

1. Observation on discoloration of O_2-saturated blood sample:— About 10 ml of blood prevented from coagulation (with ACD solution) was transferred into an Erlenmeyer flask, and shaken thoroughly to saturate it with the atomospheric O_2.

2. Hemolysate[5]:— By employing the conventional method, 1.5 volumes of H_2O and 0.5 volumes of CCl_4 were added to one volume of washed erythrocytes; the mixture was shaken vigorously (in a vortex mixer) to hemolyze the cells; the supernatant was separated by centrifugation (at 3000 r.p.m.), and used as hemolysate (hemoglobin concentration: about 10 g/dl).

For spectroscopic examination, (1) oxyhemoglobin type hemolysate: 25 μl of hemolysate+3.0 ml of Tris-EDTA borate buffer solution (0.11M, pH 8.6) (2) methemoglobin type hemolysate: 25 μl of hemolysate+3.0 ml of phosphate buffer solution (M/15, pH 6.5) + 20 μl of 5 g/dl potassium ferricyanide (mixed, and allowed to stand at room temperature for 30 minutes), (3) cyanmethemoglobin type hemolysate: 25 μl of hemolysate+5.0 ml of phosphate buffer solution (M/15, pH 6.5)+20 μl of 5 g/dl ferricyanide solution + 20 μl of 2 g/dl KCN solution, and (4) deoxyhemoglobin type hemolysate: 25 μl of hemolysate+3.0 ml of Tris-

EDTA borate buffer solution (0.11 M; pH 8.6) + one loopful of sodium dithionite (added immediately before use) were used.

For electrophoresis, (1) oxyhemoglobin type hemolysate: the hemolysate was prepared as it has been described (see above) and (2) methemoglobin type hemolysate: 1 ml of hemolysate + 10～20 μl of 5 g/dl potassium ferricyanide solution were used. (electrophoresed within 30 minutes after preparation)

3. Spectroscopy[5]. Absorption curves were traced in a Cary automatic self-recording spectrophotometer over the whole range of visible and ultraviolet light (650～260 nm).

4. Electrophoresis. Agar gel electrophoresis (pH 8.6 and pH 7.2)[6] and cellulose acetate membrane electrophoresis (pH 8.6 and pH 7.2)[7] were used.

5. Determination of Hb M content of hemolysates. Methemoglobin type hemolysate was subjected to cellulose acetate membrane electrophoresis (pH 7.2) to separate the stripe of met Hb M from that of met Hb A; the pieces of the cellulose acetate membrane that contained these stripes were cut out assembled separately, and immersed in equal volumes of phosphate buffer solution (M/15, pH 7.2) individually; absorbances E_M and E_A of the respective individual eluates were determined at wavelength of 280 nm, to estimate the percentage of Hb M content in hemolysate by the following equation:

$$\text{Hb M content} = \frac{E_M}{E_M + E_A} \times 100\ \%$$

6. Determination of Hb A_2 content.[7] Cellulose acetate membrane electrophoresis (pH 8.6) of O_2 Hb type hemolysate was employed to separate the electrophoretic stripes of Hb A_2 and Hb A + Hb M; the pieces of the membranes containing the stripes were cut out, and immersed in appropriate volumes of Drabkin's solution (the A_2 piece in 3 ml, and the A+M piece in 30 ml); absorbances of the eluates, E_{A_2} and E_{A1+M} at the Soret peak (420 nm), were measured to estimate Hb A_2 content by the following equation:

$$\text{Hb } A_2 \text{ content} = \frac{E_{A_2}}{10 \times (E_{A1+M}) + E_{A_2}} \times 100\ \%$$

7. Hb F content. Hb F content of the hemolysate was determined by Betke's method.[8]

8. Heat denaturation test of hemolysates. Carrell's isopropanol test[9] was followed.

9. Heinz body. Heinz body formation test of red blood cells was performed by the method of Dacie and Lewis.[10]

10. Fingerprinting. The green colored methemoglobin stripe was separated from the methemoglobin type hemolysate by cellulose acetate membrane electrophoresis; the membrane piece containing this electrophoretic stripe was cut out, assembled and immersed in Tris-EDTA borate buffer solution (pH 7.2; 0.065 M) for elution, and the eluates were collected by decantation to obtain purified Hb M solution. This Hb M solution was transferred into a Visking tube, and dialyzed against H_2O (at 4°C, for 2~3 days); heme was removed by Anson-Mirsky's procedure[11] to get globin as precipitate; the precipitate was washed with acetone, and dissolved in a minimal volume of water; the solution was sealed in a Visking tube, and dialyzed against H_2O at 4°C overnight; the dialysate was taken out, and freeze-dried; the freeze-dried product was separated into the α and β chains by urea-CMC column chromatography.[12] These chains were then aminoethylated[13] separately, and digested with trypsin; the digested product was fingerprinted by Baglioni's method.[14] The abnormal peptide spots of the fingerprint were eluted; the eluate was hydrolyzed,[15] and the hydrolysate was analyzed for amino acids in an automatic amino acid analyzer.[16]

11. Osmotic fragility of red blood cells. Osmotic fragility of red blood cells was determined by coil planet centrifugation.[17]

18. Diaphorase I activity (DPNH-met Hb reductase) of red blood cells. This enzyme activity was determined by Scott's method.[18]

RESULTS

The coagulation-prevented blood of this patient, even when given sufficient O_2 by aeration, remained to appear just like soy sauce in color. No significant change into scarlet color was observed. Heinz body formation test of red blood cells was slightly more markedly positive than with normal blood. Heat denaturation test of oxyhemoglobin type hemolysate was distinctly positive. Osmotic fragility of red blood cells was elevated.

The visible range absorption curve of the oxyhemoglobin type hemolysate (Fig. 1) presented α, β and γ peaks (575, 540 and 414 nm) as does the normal blood hemolysate, but the depression between α and β peaks was shallower and their declining slopes on both sides were higher (at wavelengths of about 518 and 600 nm) with a shoulder-like inflection at 605 nm. And the α peak appeared to be slightly too high

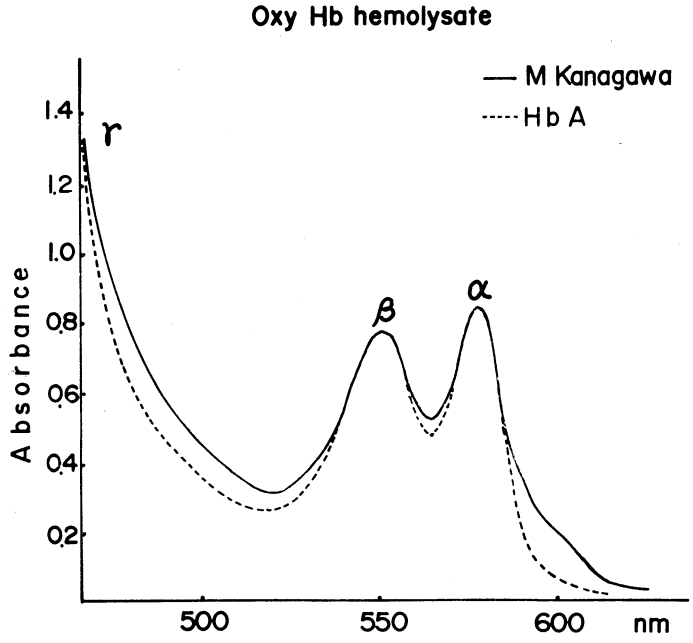

Fig. 1. Absorption curve of oxyhemoglobin type hemolysate of Hb M Kanagawa (pH 8.6)≡M Saskatoon.
Solid line: hemolysate of a patient with Hb M Kanagawa disease.
Dotted line: hemolysate of a normal subject.

for the β peak. When the height of Soret γ peak in the ultraviolet region was made comparable with that of the normal oxyhemoglobin hemolysate, γ' and ϕ peaks (at wavelengths of 346 and 274 nm) were slightly higher.

The normal acid methemoglobin hemolysate (Fig. 2) presented an absorption peak at 630 nm in the visual region, another peak at 500 nm, and a depression at 600 nm. In contrast, the hemolysate of the patient lacked the peak at 630 nm (with a shoulder at 635 nm); the depression at 600 nm was absent but, conversely, the curve protruded at this region; the peak at 500 nm shifted to 495 nm (to the shorter wavelength side); γ peak appeared at 405 nm, which was in accord with that of the normal methemoglobin type hemolysate; in the ultraviolet range, the depression at 312 nm was dislocated to 306 nm, and the elevation of 280 nm was deviated to 260 nm (to the shorter wavelength side).

The cyanmethemoglobin type hemolysate had an absorption peak at

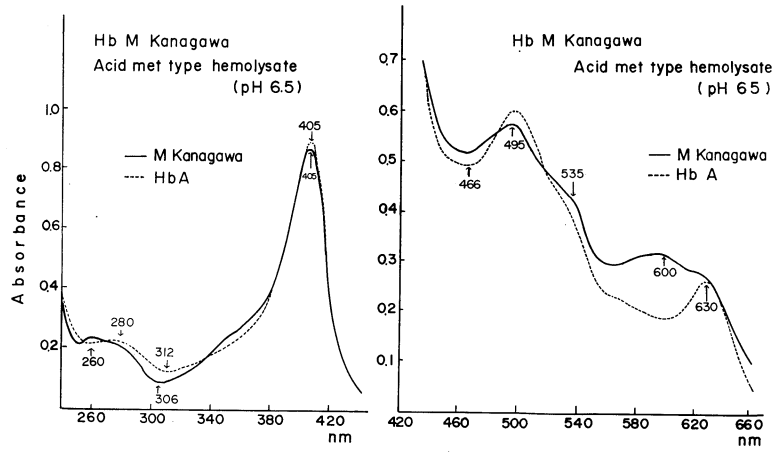

Fig. 2. Absorption curve of methemoglobin M Kanagawa (pH 6.5)≡M Saskatoon.
Solid line: acid methemoglobin M Kanagawa.
Dotted line: acid methemoglobin A.

a wavelength of 540 nm in the same way as did the normal cyanmethemoglobin hemolysate.

The deoxyhemoglobin type hemolysate of the patient gave the same absorption peak in the visual range (at 565 nm) as the normal hemolysate, but its summit was slightly flattened, and the Soret γ peak was at 428 nm, being slightly shifted to a shorter wavelength side as compared with the peak at 430 nm in the normal hemolysate.

The acid methemoglobin type purified abnormal hemoglobin presented an absorption spectrum (Fig. 3) which was markedly different from that of the normal hemolysate. There were no peak at 630 nm and no depression at 615 nm. Instead of them a sharp-pointed absorption peak was seen at 598 nm. The peak at 498 nm of the normal methemoglobin type hemolysate was deviated to 488 nm.

No abnormal electrophoretic stripe was separated from the oxyhemoglobin type hemolysate either by agar gel electrophoresis or by cellulose acetate membrane electrophoresis. Agar gel electrophoresis (pH 8.6 and 7.2) of the methemoglobin type hemolysate gave only the same electrophoretic stripe as that of the normal hemolysate. An abnormal hemoglobin electrophoretic stripe was successfully detected only by cellulose acetate membrane electrophoresis (pH 7.0). It was separated on the anode side of methemoglobin A stripe and, to be amazing, it exhibited a beautiful green color. The green colored abnormal hemoglobins accounted for 43 % of total hemoglobins.

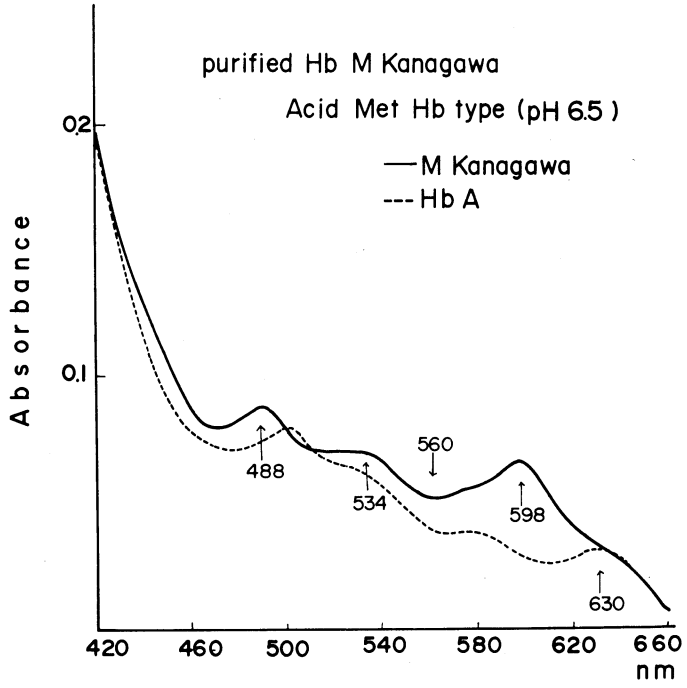

Fig. 3. Absorption curve of purified methemoglobin M Kanagawa (pH 6.5)≡M Saskatoon.
Solid line: acid methemoglobin M Kanagawa.
Dotted line: acid methemoglobin A.

The aminoethylated α chain of this abnormal hemoglobin gave a normal fingerprint. The β chain, however, presented an abnormal spot immediately below the spots of β12B$-\beta$15$+\beta$14, which was not seen in the normal β chain fingerprint. The eluate of this abnormal spot was analyzed for amino acids, and the following result was obtained: Lys 1.03=1.0; His 0.01=0; Ser 0.12=0; Glu 0.06=0; Gly 1.34=1.0; Ala 0.66=1.0; Tyr 0.77=1.0 (micromoles). Hence it was apparent that His =1.0 was lost but substituted for by Tyr=1.0 in the normal β Tp-7: Ala^{62}•His^{63}•Gly^{64}•Lys^{65}, namely, the proximal His (63 β) was replaced by Tyr.

Hb F content of this patient was 1.8 % of total hemoglobin and Hb A_2 seemed to be normal in content.

DISCUSSION

The fact that the coagulation-prevented blood of this patient, even

when saturated with the atmospheric O_2, remained just like soy sauce in color, and failed to get a scarlet hue is suggestive of two possibilities: (1) that a dark brown or blue pigment is present in the plasma and (2) that methemoglobin or Hb M is contained in the red blood cells. However, the plasma obtained from the patient's blood by centrifugation was light yellow. This finding excludes the first possibility, and the second possibility is thought to be plausible.

The oxyhemoglobin type hemolysate is abnormal because of its lack of the absorption peak at 630 nm, which characterizes methemoglobin. Methemoglobin is therefore ruled out, and Hb M seems to become most likely. In reality the acid methemoglobin type hemolysate presents an absorption spectrum entirely consistent with that of Hb M disease (absence of depression around 600 nm). The fact that DPNH-methemoglobin reductase activity in the red blood cells of this patient remained within normal range, being 128 units, tells us that methemoglobinemia is not responsible for the cyanosis of this patient.

The appearance of greenish hemoglobin stripe in the cellulose acetate membrane electrophoresis (pH 7.0) of methemoglobin type hemolysate, high content of this abnormal hemoglobin in hemolysate and positive heat denaturation test are much reminiscent of Hb M with a β chain anomaly, most probably Hb M Saskatoon[2] ($\equiv$Hb M Kurume).[19] Hb M's of α chain anomaly do not show such a high content of abnormal hemoglobin and they are greyish black in color.[1] Hb M Saskatoon is characterized by a greenish hue.[1,2] In the present study, this green hemoglobin was spectrophotometrically examined in detail not only in the visible region but also in the ultraviolet region, because its ultraviolet absorption spectrum has hitherto been little studied.

It is apparent from the fingerprint analysis of this green colored abnormal hemoglobin which was described in the foregoing section that this abnormal hemoglobin is identical with Hb M Saskatoon $\equiv$ Hb M Kurume β63 His $\rightarrow$ Tyr. It is, therefore, concluded that the patient is a case of Hb M Saskatoon disease.

The first instance of Hb M Saskatoon disease was a patient with cyanosis who was been by Baltzan and Sugarman[20] in Saskatoon (Canada) in 1950. Gerald (1958)[21] is to be credited for the first successful isolation of this abnormal hemoglobin from the hemolysate, and it was Gerald and Efron (1961)[2] who established its structural abnormality (β63 Histidine $\rightarrow$ Tyrosine).

In Japan, Kimura and his associates[22] encountered a cyanotic boy

(aged six) who was suspected of congenital heart disease in Kurume in 1960. They studied spectrophotometrically the hemolysate of the patient, and reported that the boy was a case belonging to Hb M disease namely, a patient with "neue Modifikation des Methemoglobins" which had been propounded by Hörlein and Weber (1948).[23] The abnormal hemoglobin of this patient was isolated by Yamaoka et al.[24] (1960) for the first time, but it was Shibata and his associates[19] who identified it with Hb M Saskatoon by fingerprinting and amino acid analysis. Because, until that time, this abnormal hemoglobin had been called Hb M Kurume, this name is retained as a synonym for Hb M Saskatoon even today.

Shibata et al.[25] isolated and purified Hb M Kurume from the methemoglobin type hemolysate by starch block electrophoresis (pH 7.0), and they were deeply impressed with its beautiful, green-colored electrophoretic stripe. It was further noted that in contrast to Hb M Iwate which was readily demonstrable as a chocolate brown stripe by agar gel electrophoresis (pH 7.2) of O_2 Hb type hemolysate, Hb M Kurume failed to be separated by the same procedure. Hb M Kurume was separable from Hb A by agar gel electrophoresis only after the hemolysate had been turned from O_2Hb type to methemoglobin type.[1,25]

The second case of Hb M Saskatoon disease in Japan was discovered in Yonago in 1969,[26] seven years after the discovery of Hb M Kurume case. The patient was a 12-year old cyanotic girl who came to the Tottori University Hospital. Her cyanosis was noticed half a year after birth. Shibata et al.[26] detected a greenish hemoglobin stripe by agar gel electrophoresis (pH 7.0) of the methemoglobin type hemolysate of this patient, purified the abnormal hemoglobin by starch block electrophoresis (pH 7.0), and disclosed its amino acid substitution (β63 Histidine → Tyrosine) by fingerprinting and amino acid analysis. It was at that time that they noticed the heat instability of this kind of Hb M for the first time.

No cases of Hb M Saskatoon have been recorded in Japan since then. Therefore, the case presented herein is the third instance of Hb M Saskatoon in Japan.

This abnormal hemoglobin has been reported from various regions of the world, namely, the United States of America, France, Italy, Poland, Norway, Denmark, West Germany and South Africa. It is interesting that all of these examples have been found in Caucasian races, except for those found in Japanese.[1,27] They are called by synonyms such as M Emory, M Radom, M Arhus, M Hamburg and M Chicago.

In the patient with Hb M Saskatoon disease seen by Kimura and his associates[22] in Kurume cyanosis was noticed at age 3. His parents had no Hb M. In the case discovered in Yonago,[26] the patient's father had cyanosis (although he could not be examined), and her younger brother had the same Hb M. In the case encountered at the Kanagawa Children's Medical Center described herein, the patient's father and mother were negative for the abnormal hemoglobin.

Hb M Saskatoon disease is transmitted to children from parents as Mendelian dominant trait, but in view of the fact that the abnormal hemoglobin was not demonstrable in the blood of patient's parents, the patient should be regarded to be the result of a new mutation that had occurred in the germinal cells of either the father or the mother.

Hb M Saskatoon is an Hb M of β chain anomaly. The β chain plays a role secondary to the α chain in O_2 intake into hemoglobin molecules, but plays an important role in the maintenance of the integrity of whole hemoglobin molecule.[1] Needless to say that in Hb M Saskatoon a half of its molecule pertaining to the abnormal β chains is converted to methemoglobin on account of the amino acid substitution β63 His $\rightarrow$ Tyr. The abnormal β chains present characteristic absorption spectrum similar to that of normal methemoglobin, simultaneously losing their proper function of reversible O_2 transportation. However, because its α chains are normal in structure which retain their function, Hb M Saskatoon shows normal O_2 affinity (pO_2 50 %) although its O_2 dissociation curve is less sigmoid than that of normal hemoglobin, being twisted horizontally (Hill's index, $n=1.2$).[28] Because the β63 His is not directly related to the amino acid residue which affects Bohr effect, variation in O_2 affinity of Hb M Saskatoon with shift in pH is maintained on the same level as the normal hemoglobin. Possibility of loss of heme in the abnormal β chain was studied by comparison of the β peak with the absorption peak at 275 nm, but the result was negative. No loss of heme was noted.

However, Hb M Saskatoon is unstable to some extent. Positive heat denaturation test is one of the evidences for its instability. Recently, a patient with Hb M Saskatoon disease who presented a very severe hemolytic seizure upon medication of sulfamethoxypyridazine (0.5–1.0 g daily for five to ten days) was reported by Stavem and his associates.[29] In this patient, no erythrocytes containing Heinz bodies made their appearance in the peripheral blood even at the time of the seizure. Besides this case, a patient of Hb M Saskatoon disease accompanied by reticulo-

cytosis of the level of 2.5～6.5 %, and reduction of red blood cells survival to 13 days was recorded.[27] Hb M Saskatoon disease may, therefore, be classed into unstable hemoglobin hemolytic anemia. However, Hb M Saskatoon patients hitherto encountered in Japan seldom showed signs of accelerated hemolysis (such as reticulocytosis, urobilinogenuria, increased serum bilirubins and splenomegaly); the case discovered in Kurume lacked all of such signs, and in the case encountered in Yonago positive heat denaturation test of hemolysate was the only one point suggestive of the possibility of accelerated hemolysis. The case discovered by us at the Kanagawa Children's Medical Center presented urobilinogenuria and reticulocytosis, but lacked anemia and splenomegaly. This indicates that loss of erythrocytes was sufficiently compensated for by regeneration. The accelerated hemolysis in Hb M Saskatoon may be overlooked throughout the life although cyanosis is noticed as early as immediately after birth or, at the latest, in the babyhood. Because Hb M Saskatoon disease is not a methemoglobinemia, its cyanosis can neither be alleviated by medication of large dose of ascorbic acid nor by intravenous infusion of methylene blue. The heme iron of the abnormal chain of Hb M is stabilized in oxidized state (Fe^{+++}) so firmly that it is not affected by reducing agent and DPNH and TPNH-dependent diaphorases.

REFERENCES

1) Shibata, S., Miyaji, T., Iuchi, I., Ohba, Y. and Yamamoto, K.: Hemoglobin M's of the Japanese. Bull. Yamaguchi Med. School. 14: 141-179, 1967
2) Gerald, P. S. and Efron, M. L.: Chemical studies of several varieties of Hb M. Proc. Nation Acad. Sci. 47: 1758-1767, 1961
3) Heller, P., Coleman, R. D. and Yakulis, V. J.: Plenary Session of the XIth Congress of the International Society of Haematology. Government Printer (Sydney), 1966 (pp. 427-437)
4) Shibata, S., Miyaji, T., Karita, K., Iuchi, I., Ohba, Y. and Yamamoto, K.: A new type of hereditary nigremia discovered in Akita—Hb M Hyde Park disease. Proc. Japan Acad. 43: 65-70, 1967
5) Shibata, S.: Pathological Biochemistry, Its Basic Knowledge and Its Clinical Use. Kinpodo (Kyoto, Tokyo) 1972, 1971
6) Shibata, S. and Iuchi, I.: A simple technique of agar gel electrophoresis for rapid separation of hemoglobins. Acta Haem. Jap. 24: 51-57, 1961
7) Shibata, S.: Evaluation of precision of procedures for estimation of Hb A_2 and Hb F in hemolysates (21 pages). International Symposium on Abnormal Hemoglobins and Thalassemia, Istanbul, Aug. 24-27, 1974
8) Betke, K., Marti, H. R. and Schlicht, I.: Estimation of small percentage of foetal hemoglobin. Nature 184: 1877-1878, 1959
9) Carrell, R. W. and Kay, R.: A simple method for the detection of unstable haemoglobins. Brit J. Haemat 23: 615-619, 1972

10) Dacie, J. V. and Lewis, S. M.: Practical Haematology, 3rd ed, Churchill (London) 1963
11) Anson, M.L. and Mirsky, A.E.: Protein coagulation and its reversal: the preparation of insoluble globin, soluble globin and heme. J. Gen. Phys. 13: 469-476, 1930
12) Clegg, J. B., Naughton. M. A. and Weatherall, D. J.: Separation of the α and β chains of human hemoglobin. Nature 219: 69-70, 1968
13) Jones, R. T.: Structural studies of aminoethylated hemoglobins by automatic peptide chromatography. Cold Spring Harbor Symposium on Quantitative Biology 24: 297-308, 1964
14) Baglioni, C.: An improved method for the fingerprinting of human hemoglobin. Biochim. Biophys. Acta 48: 392-396, 1961
15) Shibata, S., Miyaji, T., Iuchi, T., Ohba, Y. and Yamamoto, K.: Amino acid substitution in Hemoglobin M Akita. J. Biochem. 63: 193-198, 1968
16) Piez. K. A. and Morris, L.: A modified procedure for the automatic analysis of amino acids. Anal. Biochem. 1: 187-201, 1960
17) Kitazima, K. and Shibata, S.: Coil planet centrifugation and its application to the observation of altered membrane properties of erythrocytes in hepatobiliary disorders. J. Lab. & Clin. Med. 85: 855-864, 1975
18) Scott, E. M.: Congenital methemoglobinemia due to DPNH-diaphorase deficiency in Beutler E. (ed): Hereditary Disorders of Erythrocyte Metabolism, Grune & Stratton (New York), 1958 (p. 102)
19) Shibata. S., Miyaji, T., Iuchi, I., Ueda, S. and Takeda, I., Kimura, N. and Kodama, S.: Hemoglobin M Kurume: Its identity with Hemoglobin M Saskatoon. Acta Haem. Jap. 25: 690-694, 1962
20) Baltzan, D. M. and Sugarman, H.: Canad. Med. Ass. J. 63: 348, 1950. Quoted by Farmer, M. B., Lehmann, H. and Raine, D. N.: Two unrelated patients with congenital cyanosis due to hemoglobinopathy M. Lancet Oct. 10: 786-789, 1964
21) Gerald, P. S.: The electrophoretic and spectroscopic characterization of Hb M. Blood 13: 936-949, 1958
22) Kimura, N., Nishimoto, S., Nawata, Y., Mori, F., Kodama, S. and Nakakura, S.: Hemoglobin M disease. A case report. Jap. Heart J. 1: 456-465, 1960
23) Hörlein, H. und Weber, G.: Über chronische familiäre Methämoglobinämie, und eine neue Modifikation des Methämoglobins. Deut Med. Wschr. 73: 476-478, 1948
24) Yamaoka, K.: Hemoglobinopathy in Kyushu. Shinryo 16: 181-197 (in Japanese)
25) Shibata, S., Miyaji, T., Iuchi, I. and Ueda, S.: A comparative study of hemoglobin M Iwate and hemoglobin M Kurume by means of electrophoresis, chromatography and analysis of peptide chains. Acta Haem. Jap. 24: 486-494, 1961
26) Nakamura. K., Miura, K., Ishiwara, K., Iuchi, I., Miyaji, T., Ueda, S. and Shibata, S.: Hemoglobin M diseae discovered in Yonago—the second instance in Japanese families of M Saskatoon hemoglobinopathy. Proc. Japan Acad. 45: 613-617, 1969
27) Vella, F., Kamuzora, H., Lehmann, H., Duncan, B. and Harold, W.: A second family with hemoglobin M Saskatoon in Saskatchewan. Clin. Biochem. (Canada) 7: 186-191, 1974
28) Suzuki, T., Hayashi, A., Shimizu, A. and Yamamura, Y.: The oxygen equilibrium of hemoglobin M Saskatoon. Biochim. Biophys. Acta 127: 280-282, 1966
29) Stavem, P.. Strömme, J., Lorkin, P. A. and Lehmann, H.: Hemoglobin M Saskatoon with slight constant haemolysis, markedly increased by sulphonamides. Scand. J. Haemat. 9: 566-571, 1972