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ACCELERATED HEMOLYSIS IN Hb M AKITA DISEASE

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Abstract

In Hb M Akita disease, in addition to livid cyanosis which is peculiar to this hemoglobinopathy, signs reminiscent of accelerated hemolysis, such as increased serum hemobilirubin (1.4 mg/dl), splenomegaly (2 finger breadths), anemia (Hb=10.7 g/dl) and a rise in reticulocyte index (2.7) were observed. The anemia was thought to be in part due to shortened life span of red cells (^{51}Cr-tagging method T 1/2=11.5 days) and the sequesteration of red cells in the spleen (the spleen: liver ratio of ^{51}Cr surface count=2.5-3.0), but its main feature was sought for in an ineffective erythropoiesis of the bone marrow induced by intracellular degeneration of unstable Hb M Akita (β92 His → Tyr) and its modified pigment (Hb Akita) on the way of their production in nucleated red cells. In spite of the presence of markedly increased hematopoiesis (8 times as large as the normal; M:E ratio=0.22:1.00) deficiency of red cell supply from the bone marrow to the peripheral blood was evident (^{59}Fe red cell utilization =40.5 per cent). The distribution of the hematopoietic sites throughout the whole body was reasonably uniform. The intraerythrocytic enzyme (glycolytic system) level was rather increased, being suggestive of protective reaction in response to intraerythrocytic degradation of hemoglobin.

INTRODUCTION

Hb M Akita is an abnormal hemoglobin isolated in 1966 from the blood samples collected from the members of a family with congenital cyanosis who lived in Akita[1]. In the following year, the amino acid substitution of this hemoglobin was established[2] and identified with Hb M Hyde Park[3] which had been discovered in the United States of

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America about half a year ago. At that time Karita and Shibata saw in the proband of this pedigree (42-year-old, male) slight splenomegaly, urobilinogenuria, mild anemia, reticulocytosis and positive Heinz body formation test. Accordingly, they suspected that the Hb M Akita disease would be accompanied by accelerated hemolysis[4]. This suspicion was corroborated later, when heat denaturation test[5] of the proband's hemolysate was performed. They obtained a fairly large amount of degenerated precipitate to the same degree as seen in unstable hemoglobin hemolytic anemia.

We have, since then, desired to investigate the scale of accelerated hemolysis in Hb M Akita disease, and, at length, this year we could have the opportunity of examination of the proband by means of routine hematological tests, measurement of red cell survival (^{51}Cr-tagged cells), observation of hematopoiesis (^{59}Fe ferrokinetics), myeloscintigram using ^{99m}Tc and enzymatic study of red cells. In this paper the results of our examinations are described.

CASE REPORT

A 50-year-old man, 172 cm in height, 56-58 kg body weight, had two children (a son and a daughter) both of whom were noticed to be lividly cyanotic like he was from infancy and Hb M Akita was detected from their blood. Livid coloration was remarkable on lips and finger nails, and the ocular sclera was slightly yellow. Systolic murmur was audible at the cardiac apex area. The liver (2 finger breadths) and the spleen (2 finger breadths) were palpable. The lymph nodes were palpable in the bilateral axillary grooves. However, the patient did not complain of any disturbance in interference with daily life except for easy fatiguability. Examination of blood chemistry revealed the following abnormalities: Hb, 10.7 g/dl; the serum protein, 5.9 g/dl; icteric index, 10; serum total bilirubin, 2.4 mg/dl; (direct bilirubin, 39 per cent); serum cholesterol, 110 mg/dl; and phenol turbidity test, 9 units. The computor diagnosis based on simultaneous determination of about 20 kinds of blood chemical ingredients[6] strongly suggested that he would have a hemolytic anemia.

Examination of the peripheral blood disclosed a normocytic and normochromic anemia (RBC 301×10^4, Hb 10.7, Ht 30.4, reticulocyte index 2.7, WBC 4200, platelete 21.7×10^4) and slight poikilocytosis with some ovalocytes and sphrerocytes. Serum iron was 171 μg/dl, total iron binding capacity 259 μg/dl, serum haptoglobin 10 mg/dl (decreased markedly), and serum hemopexin 16 mg/dl.

Bone marrow (sternal) aspiration showed a hyperplastic marrow (the cell count, 969,000/mm^3) with absence of fat cells. Myeloid : erythroid ratio was 0.22 : 1 (sideroblast, 95 per cent; control, 62 per cent). A slight increase in megakaryocyte, an increase in mitotic compartment of erythroid series (rubriblast, prorubricyte and rubricyte), and mitotic pictures were seen. Serum erythropoietin was increased to 1.2 μ/ml (control, 0.39 μ/dl). Chest X ray finding and plain X ray film of the abdomen were normal, but electrocardiogram exhibited right and left ventricular high voltage. Blood pressure was 120/60 and pulse rate was 72.

METHODS

1. Erythrocytes:— a) Coil planet centrifugation[7] was employed for osmotic fragility test. b) The Dacie-Lewis method was used for Heinz body formation test[8] (modified by Yawata and Koresawa). c) To determine glycolytic enzymes and their intermediate products, the Miwa procedure[9] was used. d) In order to presume the survival and the destruction sites of red cells, the standard ^{51}Cr-tagging method and the body surface radioactivity counting method (heart, liver, spleen, etc.) were performed[10]. Radioactive sodium chromate (200 μCi) was added to the total red cell layer contained in 10 ml of blood sample and incubated at 37°C for 30 min. Then the cells were washed two times by plasma+saline mixture to remove surplus of sodium chromate, and 13 ml of the red cell suspension in saline was prepared. Ten ml of the suspension was administered intravenously. Red cell volume and plasma volume were estimated with ^{51}Cr-tagged erythrocytes[8,10].

2. Hemolysate and hemoglobin:— a) Hemolysate (Hb concentration, about 10 g/dl) was obtained by conventional technique[11]. b) Heat denaturation test was performed by Carrell's isopropanal method[12]. c) Detection and isolation of normal hemoglobins (Hb A_1, Hb A_2 and Hb F) and abnormal hemoglobins (Hb M Akita: as dark gray met Hb type, and Hb Akita: as the red O_2Hb type) was done by agar gel electrophoresis (pH 8.6; 7.0)[13] and cellulose acetate membrane electrophoresis (pH 8.6; 7.0)[14] of the O_2Hb type hemolysate and the met Hb type hemolysate.

3. Ferrokinetics:— 10 ml of the patient's plasma mixed with radioactive ferric chloride at a ratio of 1 μCi ^{59}Fe per 1 ml plasma was intravenously injected to the patient. Plasma iron disappearance (T 1/2), plasma iron turnover (PIT) and red cell uptake (utilization), etc. were determined by the standard methods[10].

4. ^{99m}Tc myeloscintigram[15] :— 14 mCi (25 μCi/kg body weight) of ^{99m}Tc-sulfur-mannitol colloid was given intravenously, and, 30 minutes later, examination of the myeloscintigram with the patient in supine position was performed (Nuclear Chicago whole-body Scintillation Camera. Collimator: High resolution) and the external counting of anterior iliac crest was done by the scintillation scanner.

TABLE 1. Experimental results

1. Erythrocyte
   a. Osmotic fragility test (CPC method)

		normal
hemolysis starting point	110mOsM	(98.2±10)
peak	94mOsM	
hemolysis end point	53mOsM	(63.8±8.2)

   b. Heinz body formation test
      peak 2.5 (control 1)
      positive cells 17.5%(control 5.5%)
   c. Intraerythrocytic enzymes & metabolic products

2,3-DPG	6032	(normal	4170 -5300)
TPI	1137	(normal	380 - 523)
GAPD	123.5	(normal	45.3 - 72.8)
GSHPX	46.4	(normal	15.5 - 27.9)
LDH	270	(normal	108 - 198)

2. Hemolysate & hemoglobins
   a. Heat denaturation test (+)
   b. Hb M Akita ·····29.3%
      Hb Akita ····· 6.8%
      Hb A_1 ····· 69.6%
      Hb F ····· 1.2%
      Hb A_2 ····· 3.1%
3. Red cell llfe span ^{51}Cr T 1/2 ·····11.5 days (normal 26-40 days)
   ^{51}Cr body surface counting ·····spleen: Liver=2.5-3.0 (normal 2.0)
   Red cell volume ·····1519 ml (normal 1210-1557 ml)
   plasma volume ·····2760 ml (normal 1470-1903 ml)
4. Ferrokinetics (erythokinetics, ^{59}Fe)
   a. plasma iron turnover·····4.6 mg/kg/day (normal 0.4-0.8 mg/kg/day)
   b. plasma iron disappearance (T 1/2)·····32 min (normal 70-140 min)
   c. Red cell utilization·····49.5% (corrected) (normal 80-95%)
   d. Surface counting·····rapid movement to the liver and the spleen, particularly to the spleen.
5. Myeloscintigram
   a. The systemic distribution of the ^{99m}Tc colloid was uniform, except for that the marrow/liver radioactivity ratio was larger in the left anterior iliac crest (3.67%) than in the right (1.82%).
   b. Hepatosplenomegaly was observed, but the radioactivity in the spleen was larger than that in the liver.

RESULTS

The results obtained were partly described in the section of the case records and additional data are presented in Table 1. Important findings are summarized as follows:

(1) Increased hemolysis:— Hyperhemobilirubinemia (1.4 mg/dl), occasionally positive urinary urobilinogen test, decrease in serum haptoglobin, and the decrease in serum hemopexin.

The life span of red cells is markedly decreased (T 1/2, 11.5 days) and an excessive accumulation of ^{51}Cr in the spleen is noted (Fig. 1).

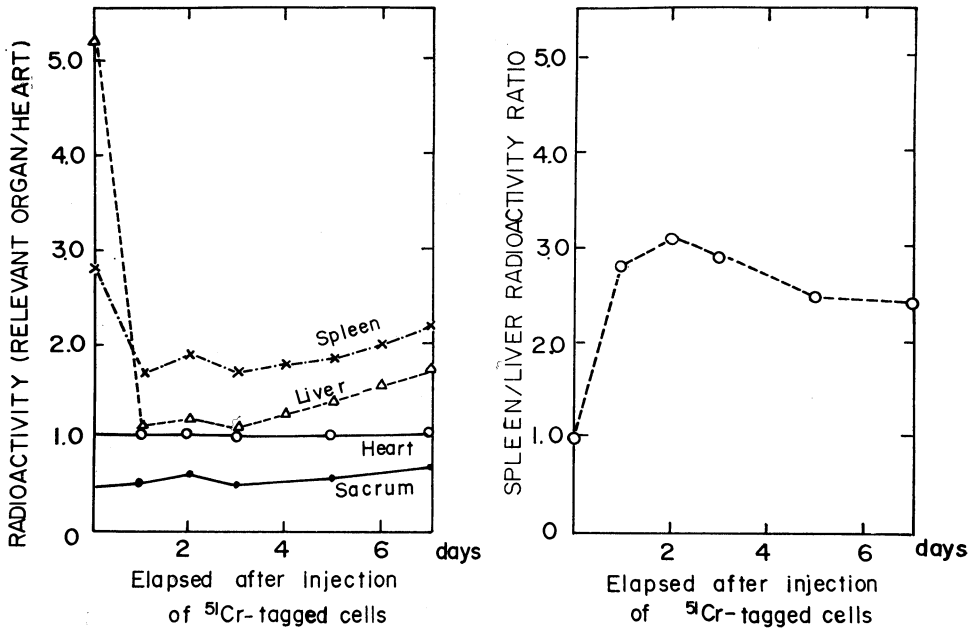

Fig. 1. Body-surface radioactivity counting over the areas of various organs after intravenous injection of ^{51}Cr-tagged erythroccytes to a patient with Hb M Akita disease. The count over the precordial region was regarded to be 1.0 as reference standard so that those over the areas of other organs may be expressed in terms of ratio (relevant organ/heart).

^{59}Fe is taken up into the bone marrow quickly, but later it accumulates relatively rapidly in the spleen and the liver, particularly into the liver (Fig. 2). In accordance with this finding the spleen is enlarged (2 finger breadths) and hepatosplenomegaly is demonstrable by ^{99m}Tc myeloscintigram (Fig. 3).

(2) Increased hematopoiesis:— In peripheral blood a slight increase in

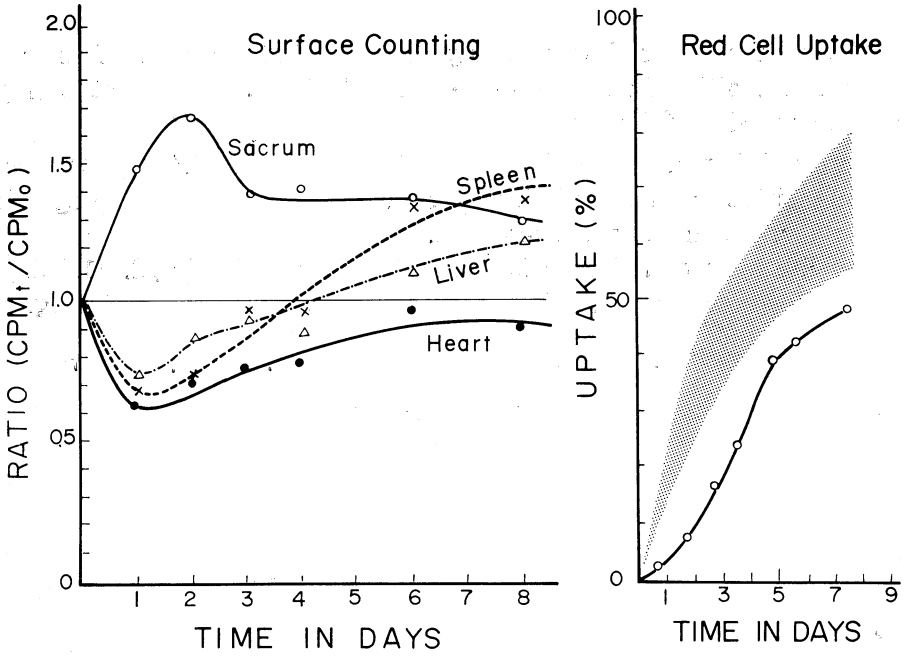

Fig. 2. Body-surface radioactivity (^{59}Fe) counting in ferrokinetic study of a patient with Hb M Akita disease (left) and his red cell uptake of ^{59}Fe (right). The surface radioactivity (^{59}Fe) at time t (CPMt) was expressed as a ratio to extrapolated zero-time radioactivity (CPMo). Uptake (%) refers to red cell volume (ml) × cpm/ml red cells ×100÷ total radioactivity injected (cpm). Shaded area indicates the normal range of uptake.

reticulocyte index (2.7) is observed and the plasma iron disappearance of ^{59}Fe is three times as rapid as normal. The plasma iron turnover (total erythropoiesis) is remarkably increased (7-8 times) (Fig. 2). Fat/cell ratio is zero in the sternal bone marrow aspirate and the M : E is decreased to 0.22 : 1, being indicative of erythroid hyperplasia of bone marrow which is nearly uniformly distributed all over the body (^{99m}Tc myelogram) (Fig. 3)

(3) Unbalance between hemolysis and hematopoiesis :— The body hematocrit is around the upper limit of normal range (1.5 l) without any indication of diminution. However, it is apparent that the patient has anemia (Hb 10.7 g/dl) and it is attributed to an increase in the plasma volume of the whole body (1.5 times as large as the normal). The red cell utilization of ^{59}Fe is poor (about 50 per cent of normal by the ferrokinetics).

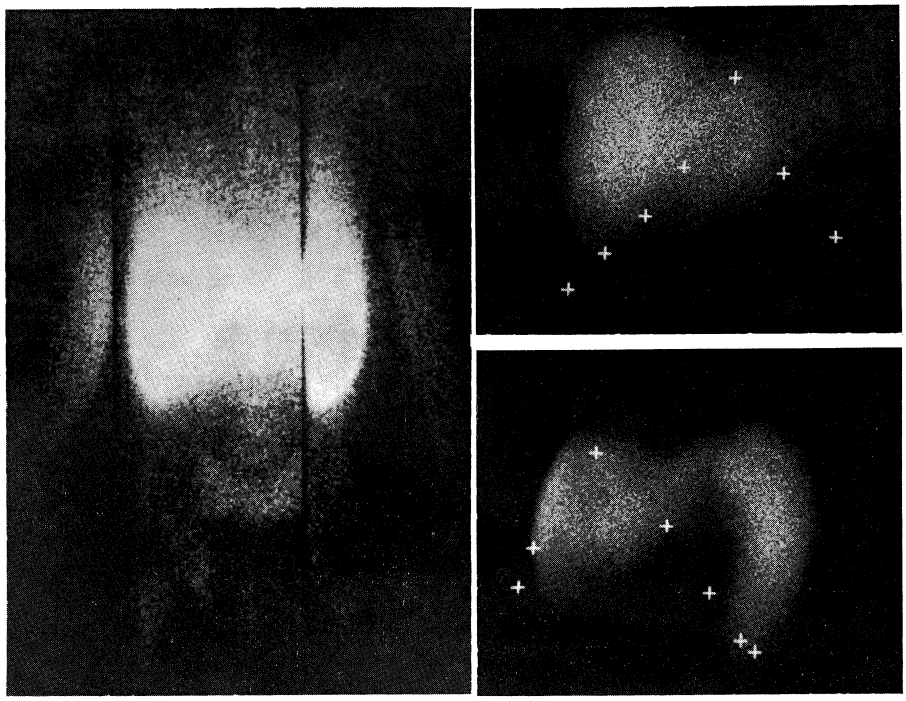

Fig. 3. ^{99m}TC myeloscintigram. *Left* :···whole body.
Right :···Hepatosplenic region.

(4) Abnormalities of erythrocytes :— Examination of the erythrocyte osmotic fragility by coil planet centrifugation reveals a hemolysis band in the coil spread both to the higher and to the lower osmolar sides, suggestive of the concommitant existence of red cells with increased and decreased fragilities (Fig. 4). It was worthy of special mentioning that the hemolysis band of the higher osmolar side was not scarlet-red in color but dark brown.

The red cells of the patient are liable to form Heinz bodies by addition of oxidizing reagents (Fig. 5) and they possess intermediate products of glycolytic enzymes larger in amount than the normal red cells, mirroring an abnormal metabolic state of increased activity. For instance, 2, 3-diphosphoglycerate (2, 3 DPG) is elevated up to 1.3 times as high as the normal; the activities of triosephosphate isomerase (TPI), glyceraldehyde phosphate dehydrogenase (GAPD), glutahione peroxidase (GSHPX) and lactic dehydrogenase (LDH) are increased to 1.5-2.0 times the normal.

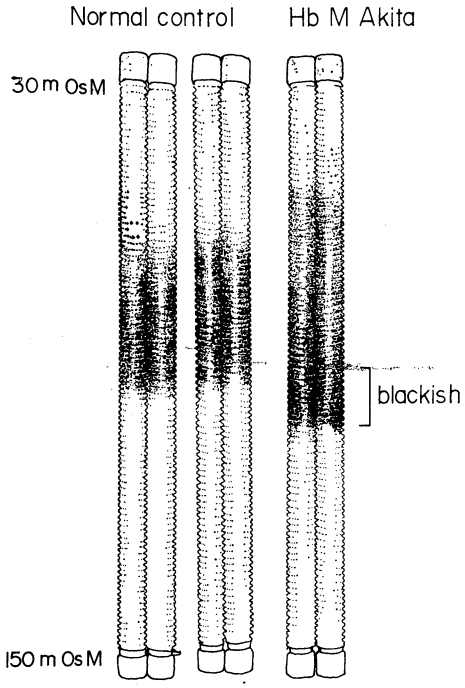

Fig. 4. Coil planet centrifugation of that patient's blood in comparison with that of a normal subject.
Note that in the patient's blood the hemolysis band is extended over the limits of normal range on both the hyper and the hypo-osmolar sides. The band is apparently blackish in the hyper osmolar portion and red in the hypo-osmolar portion, if it is shown in colored photograph.

(5) Hemoglobins:— The ratios of Hb A_2 and Hb F in the hemolysate are normal. However, by electrophoresis (pH 8.6) of the O_2 Hb hemolysate, a minor hemoglobin component which is as red as Hb A_1 is isolated between the electrophoretic stripes of Hb A_1 (Hb M Akita is concealed in this stripe) and of Hb A_2. This is called Hb Akita. The electrophoretic stripe of met Hb M Akita (dark brown in color) is demonstrable to the cathodic side of the met Hb A_1 stripe (met Hb Akita is concealed in this) only by use of electrophoresis (pH 7.0) of the hemolysate of the met Hb type. Hb M Akita and Hb Akita accounted for about 30 per cent and about 7 per cent of total hemoglobins, respectively. Precipitate is produced by Carrell's isopropanol heat denaturation test of the hemolysate, and Hb Akita (red) isolated and purified by electrophoresis (pH 8.6) of O_2 Hb type hemolysate becomes muddy forming fine precipitate in a Visking tube during the process of dialysis against water.

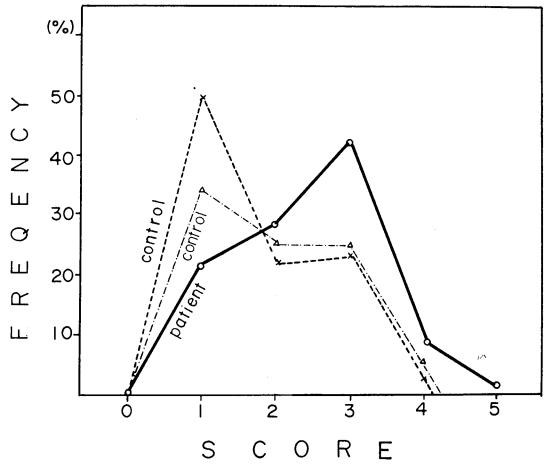

Fig. 5. Heinz body formation in patient's blood in comparison with that in a normal subject.
Note the difference between them in the number of erythrocytes containing varied numbers of Heinz bodies.

DISCUSSION

As described in the foregoing section, the patient exhibits major findings of the diagnostic criteria of hemolytic anemia (hemobilirubin $>$ 1 mg/dl, the reticulocyte index $>$ 2, blood Hb $<$ 12.5 g/dl) and its common signs (splenomegaly, increase in urinary urobilinogen, erythroid hyperplasia of the bone marrow, etc.). As regards specific findings, reduction in life span of red cells (T 1/2 $<$ 14 days) and positive detection of abnormal hemoglobins which are unstable, is observed. Therefore, it is without doubt that the patient has a hemolytic anemia. So, in this section we will discuss the mechanisms of the hemolytic anemia of this patient.

Of course, the production of Hb M Akita and Hb Akita, both of which are labile, is involved in the causation of this hemolytic anemia. Hb M Akita is an abnormal hemoglobin consists of a molecule of $\alpha_2 \beta_2^M$, with abnormal β^M chain in which the proximal His ($\beta 92$) is substituted for by Tyr. The heme iron of the β^M chain is oxidized and cannot reversibly combine with O_2, thus being incapable of transporting oxygen. Its absorption curve is similar in shape to met Hb rather than to O_2 Hb. A detailed examination of the absorption curve of Hb M Akita over the range from visible to ultraviolet regions shows a finding suggestive of partial loss of heme in the abnormal β^M chain.

In fact, Greer[16] purified this abnormal hemoglobin (Hb M Hyde Park which was obtained from the United States of America) and crystallized it in deoxy-type. He demonstrated by its X-ray crystal analysis that 20-30 per cent of the β^M chain missed heme. As heme plays an important role in stabilizing the three-dimensional structure of both α- and β- chains as their axis-shaft, it is readily presumed that, in Hb M Akita (or Hb M Hyde Park), the whole hemoglobin molecule would become fragile and prone to denaturation because of the labile conformational structure of the abnormal β^M chain. In the previous communication[17] it was poiuted out by us that Hb M Akita was unstable. In the present study this characteristic property was reconfirmed by Carrell's isapropanol test[12].

With regard to Hb Akita (red), we suspect a modified pigment of Hb M Akita in which both of the two β^M chains have missed their hemes. It becomes muddy during the process of purification by dialysis after its electrophoretic isolation.

Special attention should be paid to the fact that, in our patient, plasma iron disappearance was rapid and plasma iron turnover increased to 8 times as much as the normal. This indicates that the total erythropoiesis in the bone marrow increased to the maximum which a normal subject can achieve. Examination of the ^{99m}Tc myelogram also supports this supposition. One would expect that plethora should appear by such an increased hematopoiesis. However, the patient was not polycythemic, but anemic. The plausible explanation for this contradictory phenomenon is that the red cells containing labile hemoglobins i. e. Hb M Akita and Hb Akita, are lost or vanished in the bone marrow without attaining to the status sufficiently mature to be sent to the peripheral blood (ineffective erythropoiesis), and even when they could mature and flow in the peripheral vascular system they would be easily caught and destroyed by the spleen before completion of the normal life span of 120 days. Injury to the membrane of erythrocytes storing these labile hemoglobins due to products from hemoglobin degeneration will be the essential cause. In reality, coil planet centrifugation of the patient's blood sample reveales increased osmotic fragility of whole erythrocytes, and successfully separates two populations of erythrocytes which are represented by a dark gray red portion (at the higher osmolar side) and a red portion (at the lower osmolar side) of the hemolysis band in the coil. Erythrocytes hemolysed in the dark gray red portion are supposed to have Hb M Akita in a larger amount than those hemolysed in the red portion.

Heinz body formation test also provides evidence for hemoglobin instability.

The presence of hepatosplenomegaly demonstrable by ^{99m}Tc scintigraphy and rapid accumulation of ^{51}Cr in the spleen indicates accelerated hemolysis in the liver and the spleen. The short life span of ^{51}Cr-tagged erythrocytes (T 1/2, 11.5 days) will be accounted for by hypersplenism to a certain extent. The hypersplenism is, of course, not markedly advanced, because the leukocyte and the thrombocyte counts in the peripheral blood still remain within the normal range.

It is worthy of mentioning that ^{59}Fe utilization or uptake by red cells is decreased to about 50 per cent of the normal in the ferrokinetics study. This finding is completely contradictory to the remarkable increase in the total erythropoiesis evidenced by the increased plasma iron turnover. Therefore, we cannot but take ineffective erythropoiesis into serious consideration as an important cause of anemia of this patient, although poikilocytosis is not so evident (small number of ovalocytes and spherocytes are observed in the peripheral blood). It is supposed that as soon as Hb M Akita and Hb Akita, which are labile, appear in the nucleated erythrocyte, they undergo denaturation and the resultant product injures the cytoplasm and the membrane of the nucleated erythrocytes which are growing to maturation. These cells mitigate the injury by activation of its glycolytic enzyme system, but in case that it ends in vain they are decomposed in immature state in the bone marrow before reaching the peripheral blood, thus without making any contribution to the incrase in peripheral erythrocyte count. This is the reason why the reticulocyte index is not remarkably elevated in spite of the presence of accelerated hemolysis. The ineffective erythropoiesis may be larger in scale than the sequesteration and destruction of red cells in the liver and spleen.

Hemoglobin carries oxygen from the lungs and liberates it in the peripheral tissues. The erythrocyte 2, 3 DPG facilitates the liberation of oxygen from hemoglobin, thus rendering the supply of oxygen to the tissue easier. The increase in intraerythrocytic 2, 3 DPG observed in this patient is favorable for alleviation of tissue oxygen deficit due to anemia, but unfavorable for inducing the stimulus from the tissues to erythropoietin production. However, Hb M Akita itself possesses an O_2-transporting capacity only half as much as that of normal hemoglobin and, in addition, it occupies as much as 30 per cent of total hemoglobins. These factors and a mild decrease in the total hemoglobin concentration

cause O_2 deficit in the peripheral tissue and increased production of erythropoietin and accelerate hematopoiesis in the bone marrow to a maximum level i. e. eight times as high as the normal, although erythropoiesis ends in ineffective one for the most part on account of the damage to the red cells (nucleated and without nucleus) by the decomposition product of the abnormal hemoglobins.

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