Demonstration of Metabolic Anomaly in Congenital Nonspherocytic Hemolytic Anemia and Beneficial Effect of Vitamin B₁₂ upon It

By

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Since the first description of "a new type of hereditary hemolytic jaundice without spherocytosis" by Haden in 1947¹, similar cases have been reported by several authors under various titles such as "hereditary nonspherocytic hemolytic anemia", "atypical congenital hemolytic anemia", "nonspherocytic chronic hemolytic anemia", "familial nonspherocytic hemolytic anemia", "chronic familial nonspherocytic anemia", or "congenital nonspherocytic hemolytic anemia".²³¹³

This type of hemolytic anemia has several features in common. Jaundice or anemia usually appears soon after birth, requiring differentiation from erythroblastosis fetalis or other well-defined causes of congenital hemolytic process. Spherocytes are not found in the peripheral blood. Osmotic and mechanical resistance of the erythrocytes is normal. In most cases, no morphological anomalies of erythrocytes are observed. The anemia tends to be chronic. Hepatosplenomegaly is often present, but the course is not altered by splenectomy.⁴⁵

The pattern of inheritance of the disorder is thought to be Mendelian incomplete dominance, although conclusive pattern is not yet obtained.

CASE REPORT

A 40-day-old girl was admitted to Pediatric Clinic, Tohoku University Hospital, because of severe, prolonged jaundice from 4th day of life. The family history revealed that an elder brother died of severe jaundice on 26th day after birth and that a maternal first cousin died likewise of jaundice in early infancy (Fig. 1). The parents of the cousin were consanguineous. There was no history of jaundice or anemia in the relatives on the paternal side. The parents of the patient in question were not consanguineous, and appeared to be healthy without past history of jaundice. An uneventful full-term pregnancy and spontaneous delivery were noted. No Rh incompatibility between the patient and the mother.
could be demonstrated. The patient was of type A, CD_e/CD_e and the mother was of type O, CD_e/CD_e.

Examinations showed a well developed infant who had however remarkable jaundice and hepatosplenomegaly. The liver with smooth surface was palpable 4 cm below the costal margin on the right midclavicular line, and the spleen was palpable 1.5 cm below the left costal margin. Otherwise, no abnormal findings were present.

Laboratory data: Examination of blood showed a hemoglobin of 14.6 g/100 ml, R.B.C. 3.8 M/mm³, W.B.C. 9400/mm³, hematocrit 36%, color index 1.25, volume index 1.03, MCV 93.0 µ³, MCH 37.7 γγ, MCHC 40.5%, MCD 7.31 µ, and MCT 2.22 µ. Reticulocyte count was 42%. No normoblasts were found in the peripheral smear. There were no spherocytes or any other morphological anomalies of erythrocytes. The differential counts of leukocytes were; lymphocytes 74%, neutrophils 20%, monocytes 3.5%, eosinophils 2.0%, and basophils 0.5%. The platelet count was 235,000. Bleeding time was 1 minute 30 seconds and coagulation time was 5 minutes. The osmotic fragility test also was within normal limits, showing initial hemolysis in 0.42% saline and complete hemolysis in 0.32% saline. Both direct and indirect Coombs' tests were negative. Wassermann's reaction of both the patient and the mother was negative. The icterus index was 94 units. The Hijmans van den Bergh reaction was negative for direct and remarkably positive for indirect. Total concentration of bilirubin in the serum was 10 mg/100 ml with the indirect fraction of 7.9 mg/100 ml. The liver function tests, including thymol turbidity test, Takata's reaction, bromsulphalein test and cobalt reaction, were all within normal limits. The urine contained no bilirubin, but increased urobilinogen. Sugar and protein were negative in urine. The feaces were yellow in color and were said to have never been acholic. Urobilinogen excretion in feaces was increased. E.K.G. and X-ray of the chest revealed no abnormalities.

Basing upon the above-mentioned results, the diagnosis of congenital non-
spherocytic hemolytic anemia was established.

Family studies: The mother showed a mild anemia, R.B.C. 3.5 M/mm³, hemoglobin 12.0 g/100 ml and hematocrit 35.5%, otherwise normal in hematological data. The father showed no hematological abnormalities.

Course of the patient: The treatment with intravenous injection of 20 ml of 20% glucose and intramuscular injection of vitamin B₁₂ (50 γ) were carried out daily since the establishment of diagnosis. Jaundice improved gradually and was scarcely recognized at the end of the third week of treatment. At that time, laboratory data also showed an improvement; icterus index decreased to 23 and total concentration of bilirubin diminished to 1.6 mg/100 ml with the indirect fraction of 1.0 mg/100 ml. Urobilinogen excretion in urine and feces returned to normal. These results might indicate subsidence of hemolytic process. At present, two months after the discontinuance of the treatment, the recurrence of jaundice has not been observed.

Special studies: The determination of the content of reduced glutathione (GSH) in the erythrocytes was performed by the method of Gruenert and Phillips. The reduced glutathione stability of the erythrocytes was determined by incubation of whole blood with acetylphenylhydrazine (APH) as described by Beutler et al. The activity of glucose-6-phosphate dehydrogenase (G6PD) was assayed by the method of Zinkham et al. Vitamin B₁₂ in blood was estimated microbiologically using Lactobacillus leichmannii.

The content of GSH and the GSH stability in erythrocytes from the patient, the parents and some healthy subjects of the same age, were shown in Table I.

Table 1. Glutathione Studies in Erythrocytes from the Patient and the Parent.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GSH* (mg/100ml R.B.C.)</th>
<th>GSH (mg/100 ml R.B.C.) After 2 hours of incubation with APH**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy infant</td>
<td>A 90.3</td>
<td>83.5</td>
</tr>
<tr>
<td>(1 to 2 months of age)</td>
<td>B 89.0</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td>C 76.5</td>
<td>74.0</td>
</tr>
<tr>
<td></td>
<td>D 92.5</td>
<td>82.5</td>
</tr>
<tr>
<td>Healthy adult</td>
<td>E 67.5</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>F 70.5</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>G 76.2</td>
<td>75.0</td>
</tr>
<tr>
<td>Patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>On admission</td>
<td>43.6</td>
<td>12.8</td>
</tr>
<tr>
<td>After VB₁₂ therapy</td>
<td>54.0</td>
<td>32.5</td>
</tr>
<tr>
<td>Mother</td>
<td>59.5</td>
<td>40.8</td>
</tr>
<tr>
<td>Father</td>
<td>72.0</td>
<td>66.3</td>
</tr>
</tbody>
</table>

* GSH: Reduced glutathione, ** APH: Acetylphenylhydrazine.
Fig. 2. The glutathione stability test.

Fig. 3. Glucose-6-phosphate dehydrogenase activity.
and Fig. 2. The erythrocytes of the patient had a low level of GSH as compared with that of the controls. On the GSH stability test, GSH in erythrocytes from the patient showed a remarkable drop when incubated with APH, while GSH in erythrocytes from the controls remained almost unchanged after the incubation. The erythrocytes from the mother showed a relatively low content of GSH as well as decreased stability of GSH to a milder degree.

The erythrocytes from the father showed normal results both in GSH content and in the GSH stability test.

The results of assay for G6PD activity in erythrocytes were shown in Fig. 3. The erythrocytes from the patient showed a very low activity as compared with that from the controls. The erythrocytes from the mother showed a moderate degree of deficiency of G6PD, while those of the father were within normal limits.

![Graph showing the effect of vitamin B12 on G6PD activity](image)

**Fig. 4.** Effect of vitamin B12 on glucos-6-phosphate dehydrogenase activity.

After the treatment with vitamin B12 for three weeks, GSH in erythrocytes from the patient showed an increase from 43.6 to 54.0 mg/100 ml of erythrocytes and an improvement of the GSH stability test, while the activity of G6PD remained in low level as observed in the pre-treatment stage. The concentration of vitamin B12 in the blood of the patient was 0.42 mγ/ml, within normal limits on admission and showed a marked rise 13.55 mγ/ml during the treatment with vitamin B12.
The effect of vitamin B$_{12}$ on the activity of G6PD in erythrocytes was investigated by adding 1 $\mu$g of the vitamin to in-vitro assay system of the enzyme. The addition of vitamin B$_{12}$ showed no significant effect on the activity of G6PD in erythrocytes from the patient as well as the control (Fig. 4).

**DISCUSSION**

Recently a new field of research has been developed by the studies on the metabolism and enzymatic activity of the erythrocytes in regard to the pathogenesis of hemolytic process. Beutler and other investigators$^{16,17,18}$ elucidated that drug-induced hemolytic anemia was due to abnormality in erythrocyte metabolism. They found that the level of GSH in erythrocytes from drug-sensitive individuals was lower than that in normal and that GSH content in the erythrocytes showed a remarkable drop when these erythrocytes were incubated with APH in vitro.$^{19,20}$ Furthermore, it was demonstrated by Caros$^{21}$ that these abnormalities of glutathione metabolism were related to a deficiency of G6PD in the erythrocytes of drug-sensitive individuals.

![Glucose metabolism in erythrocytes](image)

**Fig. 5. Schema of glucose metabolism in erythrocytes.**

The hemolytic process and its relation to these three abnormalities; low content of GSH, decreased GSH stability, and deficiency of G6PD in the erythrocytes, has been explained as follows (Fig. 5).

Although biochemical mechanism of hemolysis is not enough understood, there are several evidences showing that GSH plays an important role in the
maintenance of structural integrity of erythrocyte. Benesch observed that the hemolysis induced by organic mercury compounds was protected by the previous addition of GSH in in-vitro experiments. Fegler showed an inverse correlation between GSH content of horse erythrocytes and rate of spontaneous hemolysis of them. Since GSH is thought to activate a wide variety of enzyme systems, concerning carbohydrate, fat, and protein metabolism, by protecting the enzymes against their oxidation, it is reasonable to presume that GSH is essential for the viability of the cell and for the maintenance of structural integrity of cell. The fact that glutathione exists exclusively inside the cell might support the concept mentioned above. In normal erythrocytes the maintenance of GSH is thought to be dependent on the oxidative metabolism of glucose. The conversion of oxidized glutathione (GSSG) to GSH requires the participation of reduced triphosphopyridine nucleotide (TPNH) which is generated from TPN by G6PD in the process of conversion of glucose-6-phosphate to 6-phosphogluconate. Therefore, the deficiency of G6PD results in limitation of conversion of GSSG to GSH (Fig. 5). When the individuals having a defect in G6PD in erythrocytes are exposed to a certain drug, the manifestation of hemolytic anemia is thought to take place by the marked decrease of GSH in erythrocytes as a consequence of aggravation of preexisting metabolic disturbance. The enzyme defect in drug-induced hemolytic anemia is said to be determined genetically. The agents, which can induce this type of hemolytic anemia, include primaquine, quinidine, sulfanilamide, nitrofurantoin, furazolidone, naphthalene, phenacetin, and fava bean. Irrespective of the kind of these drugs, the actual cause of hemolysis of sensitive individuals should be attributed to the deficit in G6PD in the erythrocytes. Larizza has proposed to designate “enzyme-deficiency hemolytic anemia” for this type of the disorder induced by pharmacological or vegetable agents.

Recently, Shahidi and Diamond reported that the same enzyme deficiency, as observed in the drug-induced hemolytic anemia, was found in two siblings of congenital nonspherocytic hemolytic anemia without any exposure to drugs. They described that the erythrocyte deficient in G6PD did not necessarily require the injurious effect of a drug to undergo hemolysis.

We suppose that both so-called congenital nonspherocytic hemolytic anemia and drug-induced hemolytic anemia are considered as the same entity belonging to an “inborn error of metabolism” and that the former is the case in which the manifestation of hemolysis develops soon after birth without exposure to drug and the latter is the case in which drug accentuates a preexisting metabolic disturbance to lead the manifestation of hemolysis.

We could demonstrate a lower content of GSH, its instability by incubation with APH, and a deficiency of G6PD in erythrocytes in our infant diagnosed as congenital nonspherocytic hemolytic anemia. This was the first case reported
in Japan of hemolytic anemia in which this type of enzymatic abnormality in the erythrocytes was demonstrated.

It is of interest that jaundice of our patient showed a marked improvement by the treatment with massive dose of vitamin B₁₂. Not only the improvement of clinical symptoms but a rise of GSH content and improvement of GSH instability in erythrocytes were observed in convalescence, while the activity of G6PD remained in a low level in spite of the treatment. The beneficial effect of vitamin B₁₂ on hemolytic jaundice in our case might show a close relation between vitamin B₁₂ and glutathione metabolism. The effect of vitamin B₁₂ on the reduction of some S-S compounds to the SH-state was observed by Dubnoff with in-vitro enzyme system. Ling and Chow reported that the concentration of SH-compounds, chiefly GSH, in erythrocytes was increased by the administration of vitamin B₁₂ in vitamin B₁₂ deficient rats and in the patients with pernicious anemia. A lower content of GSH in erythrocytes was found in rats or chicks deficient in vitamin B₁₂ and the supply of vitamin B₁₂ caused an increase of GSH. Although the exact site of vitamin B₁₂ on glutathione metabolism is obscure, it has been suggested that vitamin B₁₂ serves to keep glutathione in the reduced form through its participation in reductive severance of –S–S– bond or to incorporation of glycine into glutamylcystein in glutathione formation. The deficiency of vitamin B₁₂ was not recognized in our patient since the concentration in blood was within normal limits on admission. The present data, however, indicate that massive dose of vitamin B₁₂ was effective for remission of hemolytic jaundice and that this effect might be attributed to an increase of GSH and an improvement of its instability in erythrocytes after vitamin B₁₂ treatment. In this respect, an in-vitro experiment was made to observe whether or not vitamin B₁₂ would promote G6PD activity in erythrocytes. As the result of this experiment no effect of the vitamin was recognized on G6PD activity (Fig. 4). This is in agreement with the result that the activity of G6PD remained unchanged in low level in spite of treatment with vitamin B₁₂ while a marked increase of the vitamin was observed in the blood. Although the maintenance of GSH in erythrocytes would mostly depend on the activity of G6PD, it is possible that some other mechanism might take a part in the maintenance of GSH. According to Carson, glutathione reductase can utilize DPNH as well as TPNH as coenzyme. There is a report that glutathione reductase in primaquine-sensitive erythrocytes had an increased activity rather than normal. For the explanation of the beneficial effect of vitamin B₁₂ upon hemolytic jaundice observed in the present case, the following three possibilities might be supposed: 1) Vitamin B₁₂ accelerates directly glutathione reductase, 2) vitamin B₁₂ accelerates glutathione reductase indirectly by supplementing coenzyme through DPN dependent pathway in glycolysis, or 3) vitamin B₁₂ acts on blocking the oxidation of GSH, the vitamin thus causing an increase of GSH and its stabili-
lity as demonstrated in this paper. The elucidation of these supposition should await further investigation.

Another interest of the present case is genetic pattern. It has been mentioned that the enzymatic defect in erythrocytes of this type of hemolytic anemia may be transmitted as a sex-linked gene of intermediate dominance. The demonstration of enzyme deficiency in our patient, the presence of the same lesion to a lesser degree in her mother, and the absence of the defect in her father seems to agree with the genetic concept. Although a maternal cousin (a) was probably affected by the same lesion, it is possible to presume that the lesion was transmitted not from paternal side (A) but from maternal side (A') who came of the same kinship (Fig. 1). If this supposition is correct, the genetic pattern that the gene is carried by female might be accepted. It suggests a severer lesion because of hemizygote that the brother of the patient and her male cousin (a) died in early infancy. The deficiency of G6PD was milder in our patient as compared with Shahidi's cases, brothers. This difference might be explained by sex-difference, provided the abnormality is sex-linked. As seen from the pedigree of the present case (Cf. Fig. 1), the distribution of the affected gene is not always clear because of unaccuracy of statement by the parents. It is difficult to trace the genetic transmission without biochemical examinations, as a milder degree of enzyme defect and anemia were only disclosed by examinations in the mother who appeared to be normal. The manifestation of hemolysis may depend on the severity of gene penetrance on one hand and on the opportunity such as infection or exposure to certain drugs which aggravate preexisting metabolic defect in the mildly affected case on the other hand.

**SUMMARY**

1) A case of congenital nonspherocytic hemolytic anemia was reported. A metabolic abnormality in the erythrocytes from the patient was demonstrated, consisting of a low content of reduced glutathione, an abnormality in the reduced glutathione stability test, and a low activity of glucose-6-phosphate dehydrogenase. These abnormalities were also found in the erythrocytes from the mother to a milder degree, while there was no abnormalities in the erythrocytes from the father. This is the first case in Japan, of this type of hemolytic anemia in which an enzymatic anomaly of the erythrocytes was demonstrated.

2) The treatment with large dose of vitamin $B_{12}$ brought about a relief from jaundice, when an increase of reduced glutathione content in the erythrocytes and an improvement of reduced glutathione instability were observed, although the activity of glucose-6-phosphate dehydrogenase in the erythrocytes remained in a constantly low level.

3) The in-vitro effect of vitamin $B_{12}$ on glucose-6-phosphate dehydrogenase was investigated. The relationship between vitamin $B_{12}$ and glutathione me-
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4) Genetic mode of the disease was discussed.

References

6) Feinberg & Watson, Blood, 1951, 9, 357.
9) Lipton et al., Pediatrics, 1953, 12, 384.
18) Szeinberg et al., Blood, 1957, 12, 603.
33) Zinkham & Childs, Pediatrics, 1958, 22, 461.
40) Hsu et al., Arch. Bioch. & Biophys., 1959, 84, 15.