A New Inherited Abnormality of Hemoglobin and Its Interaction with Sickle Cell Hemoglobin

By Eugene Kaplan, M.D., Wolf W. Zuelzer, M.D. and James V. Neel, M.D., Ph.D.

This report deals with a new inherited abnormality of hemoglobin encountered in American Negroes. The existence of this trait came to light in the course of extensive clinical and genetic studies regarding the sickling phenomenon. Among the individuals examined there were a number of patients with a hemolytic syndrome associated with erythrocytic sickling in whom neither the clinical, hematologic, nor genetic pattern fitted that of typical sickle cell anemia. Following the demonstration by Pauling and his associates that sickle cell hemoglobin has a characteristic electrophoretic mobility, blood from these unusual individuals and their families was subjected to electrophoretic analysis. These studies, described in detail in a separate report, disclosed the existence of a new component of hemoglobin clearly distinguishable from both normal and sickle cell hemoglobin. In the present paper the clinical, hematologic and genetic features associated with this newly recognized abnormality of hemoglobin are described.

For the time being this new hemoglobin component is designated as hemoglobin III, since it is the third type of adult human hemoglobin to be identified by electrophoretic methods, the other two being normal and sickle cell hemoglobin.*

In the individuals thus far studied the combination of hemoglobin III with sickle cell hemoglobin is associated with a mild hemolytic syndrome, erythrocytic sickling and splenomegaly. In combination with normal hemoglobin, hemoglobin III is associated with an asymptomatic carrier state without erythrocytic sickling. The homozygous condition with respect to hemoglobin III has not yet been recognized.

Methods

Sickling preparations were made with sodium metabisulfite (kindly supplied by Lilly & Co.) as a reducing agent in sealed cover glass films of capillary blood. Siderocyte preparations were made by the method of MacFadzean and Davis. The mechanical fragility of erythrocytes was determined by the method of Shen and his associates. In this laboratory, normal controls have ranged from 4 to 10 per cent hemolysis in oxygen atmosphere, and from 6 to 13 per cent hemolysis in carbon dioxide atmosphere.
The saline fragility of erythrocytes was determined by a method adapted from Ponder. In our experience the average upper limit for hemolysis is .45 per cent to .49 per cent saline, and the lower limit is .33 per cent to .28 per cent saline.

Fecal urobilinogen excretion was determined on pooled four day specimens by the method of Watson, and the results were expressed as "Hemolytic Index," using the formula of Miller, Singer and Dameshek.

Daily fecal urobilinogen excretion in mg.

where the blood volume was estimated at 7.6 per cent of the body weight.

Serum bilirubin was measured by the method of Powell.

Erythrocyte survival was measured by a modification of the Ashby technic of differential hemagglutination, based on the method of Young, Platzer and Rafferty.

CASE HISTORIES

I Family W

Family W. was studied because 2 of 3 children were found to have erythrocytic sickling and hemolytic anemia. The parents are native born American Negroes whose physical appearance suggests some admixture of Caucasian stock. The mother has the sickling trait and her hemoglobin consists of a mixture of normal and sickle cell components. The father's erythrocytes do not sickle, but contain a mixture of normal hemoglobin and hemoglobin III. The hemoglobin of the 2 older children, who have a mild hemolytic anemia and erythrocytic sickling, consists of a mixture of sickle cell hemoglobin and the new component. The hemoglobin of the youngest child is of the normal type.

Case 1. Rosetta W., is a 12 year old Negro girl who has attended the Out-Patient Clinic of Children's Hospital since infancy for an occasional mild respiratory, intestinal or skin infection. There have been no episodes of pallor, icterus or pain. Hepatosplenomegaly has been noticed since she was 2 years old. The spleen has gradually increased in size and now extends 6 cm. below the costal margin, being smooth, firm and nontender. The liver edge has remained at approximately 2 cm. below the costal margin. A functional cardiac murmur has been present since infancy in the absence of cardiac enlargement or abnormal electrocardiographic tracings. Erythrocytic sickling was first demonstrated when the patient was 4 years old during an attack of lobar pneumonia. The hemoglobin level at that time was 10.8 Gm. per cent. Since that illness she has remained in apparently good health, and is now an alert adolescent girl of normal height, weight and development. Roentgenograms of the skull and extremities have shown nothing unusual.

During the two year period of our observations, there has been a very mild normochromic anemia with minimal reticulocytosis and no elevation of leukocytes or serum bilirubin (table 1). Target cells are frequent in fixed peripheral blood films, but crescentic sickle forms and iron-staining erythrocyte granules usually present in sickle cell anemia are virtually absent. In sealed moist blood films the erythrocytes sickle rapidly with formation of long filaments. There is a moderate increase in the fecal urobilinogen excretion. Bone marrow examination reveals a moderate erythroid hyperplasia (table 2). The patient's erythrocytes are rapidly eliminated after transfusion into a normal recipient. (fig. 1) whereas normal erythrocytes transfused into the patient are eliminated at a normal rate (fig. 2). Electrophoretic study of the patient's hemoglobin revealed both the component characteristic for sickle cell hemoglobin and a new component which moves as a more positive ion than either normal or sickle cell hemoglobin (fig. 3).

Case 2. Terry W., a 10 year old Negro boy, brother of Rosetta, has been free of any complaint for the last eight years, and is now an active, alert child of normal height, weight and development. During his infancy no abdominal visceral enlargement or other abnormality was noted at repeated visits to the Well-Baby Clinic. At age 24 years he had an acute febrile illness with coryza and back pain followed two days later by marked pallor. On entry to this hospital the following day he was acutely ill, temperature 102 F., not icteric, but very pale with signs of nasopharyngitis and enlargement of both liver and spleen 3
Table 1.—Summary of Hematologic Data

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Family C:

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Key for table 1.

Hg. III, red blood cells, millions per cubic millimeter.
Hg., hemoglobin, grams per cent.
Retic., reticulocytes, per cent.
Ht., hematocrit, venous blood.
W.B.C., white blood cells, thousands per cubic millimeter.
MCV, mean corpuscular volume.
MCH, mean corpuscular hemoglobin.
MCC, mean corpuscular hemoglobin concentration.
Sickling, in moist blood films.
Target %, per cent target cells in dry blood films.
Sal. Frag., osmotic fragility, per cent saline.
M. Frag., mechanical fragility; per cent O2 in oxygen atmosphere, CO2 in carbon dioxide atmosphere.
S. Bil., serum bilirubin, milligrams per cent.
Hem. Index, hemolytic index (fecal urobilinogen excretion).

Table 2.—Bone Marrow Differentials; Per Cent Distribution

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<th>Case 6 P. C.</th>
<th>Case 7 Pr. C.</th>
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<td>Reticulum cells</td>
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<td>Stem cells</td>
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<td>Lymphocytes</td>
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<td>Erythroid Cells</td>
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<td>Pronormoblast</td>
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<td>Eosinophils</td>
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<td>0.8</td>
</tr>
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<td>Basophils</td>
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<tr>
<td>Megakaryocytes</td>
<td>Normal</td>
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<tr>
<td>Myeloid:Erythroid Ratio</td>
<td>1:2</td>
<td>1:1</td>
<td>3:1</td>
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NEW INHERITED ABNORMALITY OF HEMOGLOBIN

Hg. III, abnormal hemoglobin component by electrophoresis.
Sickle Hb., abnormal hemoglobin component by electrophoresis.
R.B.C., red blood cells, millions per cubic millimeter.
Hg., hemoglobin, grams per cent.
Retic., reticulocytes, per cent.
Ht., hematocrit, venous blood.
W.B.C., white blood cells, thousands per cubic millimeter.
MCV, mean corpuscular volume.
MCH, mean corpuscular hemoglobin.
MCC, mean corpuscular hemoglobin concentration.
Sickling, in moist blood films.
Target %, per cent target cells in dry blood films.
Sal. Frag., osmotic fragility, per cent saline.
M. Frag., mechanical fragility; per cent O2 in oxygen atmosphere, CO2 in carbon dioxide atmosphere.
S. Bil., serum bilirubin, milligrams per cent.
Hem. Index, hemolytic index (fecal urobilinogen excretion).
Fig. 1.—The survival of erythrocytes from individuals with hemoglobin III and sickle hemoglobin (Cases 1 and 6) transfused into normal recipients. Erythrocytes from 1 individual (Case 6) were transfused on separate occasions into 2 different recipients. The broken line approximates the theoretical normal curve of erythrocyte survival. %—percent survival of donor cells; days—days following transfusion.

Fig. 2.—The survival of normal erythrocytes transfused into individuals with hemoglobin III and sickle hemoglobin (Cases 1 and 6).
NEW INHERITED ABNORMALITY OF HEMOGLOBIN

em. below the costal margin. Study of his blood (the first recorded for this patient) revealed hemoglobin 3.5 Gm. per cent, red cells 1,260,000, many nucleated red cells, white cell count (uncorrected) 50,000 per mm³, and erythrocyte sickling. X-rays of the chest and skeleton revealed no abnormalities. He received several transfusions and was discharged after a rapid convalescence with the diagnosis of sickle cell anemia.

Since that illness he has remained in excellent health, entirely free of attacks of pallor, icterus or pain. At present his heart is normal in size, configuration and physical signs. The liver extends 5 cm. and the spleen 3 cm. below the costal margin, both of firm consistency and nontender. X-rays of the skull and extremities reveal no abnormalities.

The hematologic findings during the last two years have been virtually identical with those in his sister (table 1). Noteworthy is a mild anemia, with little reticulocytosis or leuko-

Fig. 3.—Longsworth scanning diagrams of the carboxyhemoglobins of five different types of individuals, after Itano and Neel (1950).
cytosis in the presence of erythrocyte sickling. Target cells are prominent in fixed peripheral blood films, but sickled forms and siderocytes are virtually absent. The electrophoretic pattern of the patient's hemoglobin was identical with that of his sister.

**Case 3.** Eric W., brother of Rosetta and Terry, is an 8 year old boy who, apart from chronic asthma, is normal both with respect to his clinical and hematologic status (table 1). Electrophoretic analysis showed his hemoglobin to be entirely of the normal variety.

**Case 4.** Mrs. W., mother of these children, is 32 years old and in excellent health. Except for erythrocyte sickling her blood is entirely normal. Electrophoretic study revealed that her hemoglobin gives the characteristic pattern for the sickle cell trait—a mixture of normal and sickle cell hemoglobin.

**Case 5.** Mr. W., the father, is a well developed and nourished Negro, aged 34 years, who has had no significant complaint or illness, and who served in the Pacific Theater during World War II. There is no history of anemia in his parents. Physical examination of this subject revealed no abnormalities and his peripheral blood is normal with respect to the

![Peripheral blood of individual with hemoglobin III trait (Case 5). Note numerous target cells.](image)

concentrations of hemoglobin, red cells, reticuloerythrocytes, leukocytes and bilirubin (table 1). On fixed stained blood films taken on numerous occasions erythrocytes of target cell shape are very prominent (fig. 4), ranging from 18 per cent to 25 per cent of the cell population. The remaining erythrocytes are within normal limits of size, shape and hemoglobin content. Erythrocyte sickling is consistently absent. The red cells are unusually resistant to hemolysis in hypotonic saline, but the mechanical fragility is normal. When transfused into a normal recipient, his erythrocytes were eliminated at an abnormally rapid rate (fig. 6). Electrophoretic study of his hemoglobin revealed a mixture of two types, the normal, and the new component present in both Rosetta and Terry.

II Family C

Family C was studied because the youngest of 3 children was found to have erythrocytic sickling and hemolytic anemia. The parents are both native born American Negroes with the physical appearance suggestive of some mixture with Caucasian stock. The mother has the sickling trait and her hemoglobin consists of a mixture of normal and sickle cell hemoglobin. The father's erythrocytes do not sickle, but contain both normal hemoglobin and hemoglobin III. The hemoglobin of the oldest child, like that of the father, is a mixture of normal and hemoglobin III. The hemoglobin of the second child is entirely of the normal
NEW INHERITED ABNORMALITY OF HEMOGLOBIN

type. The hemoglobin of the youngest child, who has a hemolytic anemia and erythrocytic sickling consists of a mixture of three components, sickle cell hemoglobin, hemoglobin III and a small fraction of normal hemoglobin.

Case 6. Pearl C., a 4 year old Negro girl, had not been ill until 2 years ago when she was seen in the dispensary because of a respiratory infection. It was found that she was severely anemic, slightly icteric and had enlargement of the spleen. During convalescence the icterus subsided and the anemia rapidly improved without transfusion. Since then she has remained free of complaints and has had no further pallor or icterus. At present she is an active well developed preschool child with no abnormalities on physical examination save for moderate enlargement of both the liver and spleen, each extending 2 cm. below the costal margin. There is no cardiac enlargement, and the skeleton is normal on x-ray examination.

For the two year period of our observation there has been a moderate normochromic normocytic anemia associated with erythrocyte sickling, mild reticulocytosis, normal leukocyte count, and no hyperbilirubinemia (table 1). Target cells are frequent in films of the peripheral blood (fig. 5), but sickled forms and iron-staining erythrocyte granules are absent. In vitro sickling is rapid and predominantly filamentous in type. Fecal urobilinogen exere

Fig. 5.—Peripheral blood of individual with hemoglobin III and sickle cell hemoglobin (Case 6). Note numerous target cells and absence of sickle forms.

tion is moderately increased. There is a moderate erythroid hyperplasia of the bone marrow. The patient's red cells are rapidly eliminated when transfused into a normal recipient (fig. 1). Normal erythrocytes have normal survival when transfused into the patient (fig. 2). The hemoglobin, on electrophoretic analysis, was found to be a mixture of three components: sickle cell hemoglobin, hemoglobin III and a very small proportion of normal hemoglobin (table 3).

Case 7. Prime C., Pearl's 6 year old brother, is a well developed child in excellent health, who has had no serious illness and no significant complaints. Physical examination and x-ray examination of his chest and skeleton reveal no abnormalities. The peripheral blood count and serum bilirubin are normal (table 1). Erythrocytic sickling is absent. On fixed blood films, the erythrocytes are round, well filled with hemoglobin and normal in size. From 3 to 5 per cent of the erythrocytes are target cells. There is increased resistance to hemolysis in hypotonic saline. The mechanical fragility is normal. The bone marrow is not abnormal (table 2), and the fecal urobilinogen excretion is not increased. The patient's erythrocytes are rapidly eliminated when transfused into a normal recipient (fig. 6). Electrophoretic study of the hemoglobin revealed a mixture of normal hemoglobin and hemoglobin III.
Fig. 6.—The survival of erythrocytes from individuals with hemoglobin III trait (Cases 5, 7 and 10) transfused into normal recipients. Erythrocytes from one individual (Case 10) were transfused on separate occasions into two different recipients.

TABLE 3.—Percentile Distribution of Hemoglobin Components*

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<th>Per Cent Distribution</th>
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<th>Sickle Hemoglobin</th>
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<tr>
<td>Normal</td>
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<td>100</td>
<td>55-76</td>
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<td>Case 2 T. W.</td>
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<td>Case 3 E. W.</td>
<td>100</td>
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<td>Case 4 Mrs. W.</td>
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<td>Case 5 Mr. W.</td>
<td>69.8</td>
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<td>Family C.</td>
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<td>Case 6 P. C.</td>
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<td>Case 7 Pr. C.</td>
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<td>Case 9 Mrs. C.</td>
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<td>Case 10 Mr. C.</td>
<td>64.7</td>
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<td>35.3</td>
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* Table adapted from Itano, H. and Neel, J. V. Proc. Nat. Acad. Se. 38: 613, 1950.

Case 8. Robert C., Pearl's 5 year old brother is entirely normal with respect to his clinical and hematologic status. Electrophoretic analysis showed his hemoglobin to be entirely of the normal variety.
NEW INHERITED ABNORMALITY OF HEMOGLOBIN

Case 9. Mrs. C., mother of these children, is 29 years old and in excellent health. Except for erythrocytic sickling her blood is entirely normal and gave the electrophoretic pattern typical for the sickle cell trait.

Case 10. Mr. C., the father, is a well developed 30 year old Negro in excellent health. His family history is noncontributory. No abnormalities are found on physical examination. The peripheral blood levels of red cells, hemoglobin, reticulocytes and leukocytes are within normal limits. A slight hyperbilirubinemia was present on two occasions. Peripheral blood films contain 8 to 12 per cent target cells. The osmotic and mechanical fragility are normal (table 1). Electrophoretic study of his hemoglobin revealed a mixture of two types, the normal and the new component. The erythrocytes were transfused into a normal recipient and were eliminated at a very rapid rate. Several months later the subject was bled again and the erythrocytes were transfused into a second normal recipient with identical results (fig. 6).

SPECIAL INVESTIGATIONS

1. Electrophoresis

The new type of hemoglobin migrates in the Tiselius apparatus as a more positive ion than either normal hemoglobin or sickle cell hemoglobin (fig. 3). This behavior characterizes yet another molecular abnormality of hemoglobin structure, distinct from the two previously identified types of hemoglobin, the normal and the sickle cell variant. The details of the electrophoretic analysis have been given elsewhere.2 The new component, hemoglobin III, was encountered in combination with sickle cell hemoglobin in Cases 1 and 2, with an additional small fraction of normal hemoglobin in Case 6; and in combination with normal hemoglobin only, in Cases 5, 7 and 10. The percentile distribution of these components is given in table 3.

The presence of hemoglobin III and normal hemoglobin in the same individual is comparable to the simultaneous presence of sickle cell and normal hemoglobin in individuals with the sickle cell trait, as demonstrated by Pauling and his associates.1 Thus far, hemoglobin III has been found only in admixture with other types of hemoglobin. As yet, the analogous situation to the usual pattern of sickle cell anemia, in which the hemoglobin is uniformly composed of a single abnormal variant, has not been recognized with respect to hemoglobin III.

2. Genetic Studies

In each of the two families one parent had the new component in admixture with normal hemoglobin, while the other parent had the typical sickle cell trait with the mixture of normal and sickle cell hemoglobin. Three types of offspring were produced by these matings (fig. 7). Two children in Family W. (Cases 1 and 2) had a combination of sickle cell hemoglobin and hemoglobin III, and one child in Family C. (Case 6) had the same mixture with an additional small fraction of normal hemoglobin. Secondly, one child in Family C. (Case 7) had a mixture of hemoglobin III and normal hemoglobin. Lastly, one child in each family had only the normal hemoglobin.

These facts indicate that the presence of hemoglobin III, like that of sickle cell hemoglobin, is determined by a gene which appears to be transmitted as a simple Mendelian dominant. The precise relationship of this gene to that responsible for the sickling phenomenon is not clear as yet. The two genes may be transmitted independently of each other, they may be linked on the same chromo-
some, or they may prove to be members of a series of multiple alleles. The method for differentiating between these possibilities has been presented elsewhere.\textsuperscript{14} The differentiation is not possible on the basis of the limited data now available.

In any event the presence of the gene for hemoglobin III appears to exert a modifying effect on the manner in which a single gene for erythrocyte sickling is expressed. Ordinarily, the presence of a single sickling gene produces only the asymptomatic sickle cell trait. The development of the hemolytic syndrome, classical sickle cell anemia, depends on the presence of a pair of sickling genes, one from each of the parents.\textsuperscript{15, 16} In our patients, however, a hemolytic syndrome associated with erythrocyte sickling was found even though only one of the parents could have transmitted a sickling gene. It is apparent that this modification resulted from the presence of the gene for hemoglobin III. Since in each family the mother contributed the sickling gene, the possibility that the hemolytic syndrome in the children was actually the result of an additional sickling gene inherited from a father other than the legal parent was considered. The result of a detailed analysis of the blood groups (table 1) did not indicate any possibility of impaternity in either family. Moreover, the distribution of the new hemoglobin in itself supports the evidence for true paternity.

Since in all the individuals studied each of the two abnormal hemoglobins appears in combination with the other or with normal hemoglobin, rather than as the sole component, it is of interest to note the quantitative relationship of the various fractions. Unpublished studies\textsuperscript{17} suggest that there are significant intrafamily correlations with respect to the percentage of sickle cell hemoglobin present as determined by electrophoresis so that the proportion of abnormal hemoglobin in a child with the sickle cell trait tends to resemble the proportion in his sickling parent.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{family_pedigrees.png}
\caption{Family pedigrees. Numbers refer to cases, table 1.}
\end{figure}
NEW INHERITED ABNORMALITY OF HEMOGLOBIN

In the case of sickle cell anemia it would seem that the two sickling genes may enhance each other beyond their simple additive effect so that the hemoglobin of such subjects may consist of 100 per cent sickle variety even though in each of the parents the particular gene resulted in considerably less than 50 per cent sickling hemoglobin. The data presented in table 3 indicate that similar conditions may govern the quantitative aspects of hemoglobin III. In the only child whose hemoglobin was found to be a mixture of the new variety with normal hemoglobin the percentage distribution was, within the limits of technical error, almost exactly that of his father.

In the three individuals whose hemoglobin proved to consist of hemoglobin III and sickle cell hemoglobin the value for each of the two fractions was at, or approached 50 per cent, even though in the parents the corresponding values were only 30 per cent. These figures suggest that the two different genes present in these subjects may potentiate each other much as the two similar genes of the sickle trait potentiate each other in sickle cell anemia. A larger experience is required to show whether this mutual potentiation is a consistent phenomenon.

3. Hematologic Observations

A. Subjects with Hemoglobin III and Sickle Cell Hemoglobin. In the patients who had inherited both the gene for sickling and that for the new component (Cases 1, 2, 6) the sickling phenomenon was invariably present in vitro. Although no sickled forms could be found in fixed blood films (in contrast to their presence in the great majority of blood films from subjects with sickle cell anemia) sickling in sealed wet preparations occurred in a manner resembling that of sickle cell anemia, and distinctly different from the uncomplicated sickle trait. While there is at present no agreement concerning differences in the rate and morphology of the sickling phenomenon between the trait and sickle cell anemia it has been our experience that in general erythrocytic sickling in sickle cell anemia is distinctly more rapid and quickly results in the formation of long filamentous forms. In the trait, under comparable conditions, holly leaf forms predominate. The erythrocytes from our patients when sealed in a moist drop with sodium metabisulfite show complete sickling in a very short time and virtually all the cells assume the filamentous shape.

In dry, stained blood films the only striking feature of the erythrocytes is the presence of numerous target cells which comprise from 45 per cent to 75 per cent of the red cell population. In this respect, too, the resemblance to classical sickle cell anemia is marked. In other respects the erythrocyte morphology is remarkably normal. There is no appreciable anisocytosis, poikilocytosis or polychromasia. The cells are normochromic and the corpuscular constants are within the normal range for children. There is only slight elevation of the reticulocyte count. Nucleated red cells are extremely rare. In ordinary Romanovsky stains no erythrocyte inclusions are seen.

The number of siderocytes (erythrocytes with iron-staining inclusions by the Prussian-blue technic) was not increased as compared with normal controls. This finding stands in contrast to the increased frequency of siderocytes in the majority of sickle cell anemia patients. A systematic study of siderocytes in sickle cell disease now in progress in our laboratory indicates that siderocytosis
is a fairly constant feature in sickle cell anemia, but is not present in the simple sickle trait.

The resistance of the erythrocytes to hypotonic saline is significantly increased. The figures are within the range encountered in sickle cell anemia. The erythrocytes have a normal mechanical fragility in an oxygen atmosphere. After saturation with carbon dioxide gas, their mechanical fragility is significantly increased. This behavior would seem to be dependent upon the sickling phenomenon since in our own experience it is equally characteristic of sickle cell anemia and sickle cell trait.

As judged by the usual criteria for in vivo hemolysis, reticulocytosis, bilirubinemia and increased fecal urobilinogen excretion, only a mild hemolytic disturbance was demonstrable in our patients during the period of our study. The reticulocyte and serum bilirubin levels were only slightly elevated, or at the upper limits of normal. Fecal urobilinogen excretion was measured in 2 of the 3 patients and was found to be only moderately increased. The hemolytic indexes were 19 and 24 respectively, figures which lie definitely above the normal range of 2 to 7 in children. The increase in fecal urobilinogen excretion might reflect a metabolic shunt such as has been demonstrated in sickle cell anemia.

The possibility that part of this increment might be due to such a mechanism was not excluded with the methods available to us. It is unlikely, however, that a metabolic shunt explains all of the increase, since there is marrow erythroid hyperplasia which suggests compensatory activity for increased red cell destruction. The myeloid-erythroid ratio in the bone marrow of the 2 patients studied was 1:1 and 1:2, values well beyond the normal range, although not as extreme as in the usual cases of sickle cell anemia.

Erythrocyte survival studies were carried out in Patients 1 and 6. The survival of normal cells transfused into both patients was normal. The survival of cells from either patient transfused into normal recipients was markedly shortened, with a half-life of twelve days in Case 1, and six and eighteen days respectively on separate occasions in 2 different normal recipients in Case 6 (figs. 1, 2). This behavior indicates an intracorporeal erythrocyte defect comparable to that of sickle cell anemia. Shortened erythrocyte survival is not observed in the sickle cell trait. It is therefore noteworthy that it could be demonstrated in these patients who had inherited only a single sickling gene and who in this respect are comparable to individuals with the sickle cell trait. Since in addition to sickle hemoglobin the erythrocytes of our patients contain the new component it is evident that the shortened survival results either from the interaction of the two abnormal hemoglobins or is related to the presence of hemoglobin III per se. The data to be presented in the following section tend to support the latter view.

B. Subjects with Hemoglobin III and Normal Hemoglobin. These individuals (Cases 5, 7, 10) had no symptoms related to anemia and exhibited no unusual physical findings. The red blood cell and hemoglobin values were in the optimal range for their age and sex. In one instance, Case 10, slight elevation of the serum bilirubin was present on two occasions due to an increase in the “indirect” fraction; the reason for this finding was not apparent. In general there was no evidence of a hemolytic process. In 1 individual both a bone marrow puncture and a fecal urobilinogen determination were performed, with entirely normal results.
Repeated tests for erythrocytic sickling were uniformly negative. Erythrocyte inclusions, Howell-Jolly bodies, and nucleated red blood cells were uniformly absent. The only morphologic abnormality of the erythrocytes was the presence of numerous target cells in fixed blood films (fig. 4). These cells were seen amidst a normocytic normochromic red cell population and themselves neither appeared appreciably hypochromic nor did they show any noticeable deviation from the normal diameter. In repeated counts target cells consistently ranged between 16 to 25 per cent in Case 5, and between 8 to 12 per cent in Case 10. These figures are well beyond the normal range. In the third subject, Case 7, 3 to 5 per cent target cells were present. In our experience with a large number of normal non-anemic subjects the usual frequency of target cells is less than 1 per cent, but occasionally we have observed up to 2 to 3 per cent target cells in apparently normal white subjects, and up to 3 to 4 per cent target cells in apparently normal Negroes. The incidence of target cells in Case 7 is therefore probably significant, or at least at the upper limits of normal. Further studies on relatives of Family W. which will be fully described at a later date disclosed a high incidence of target cells, in the absence of anemia, in one of the brothers of Mr. W. and in 2 of his children.

The resistance of the erythrocytes to hypotonic saline was increased in two subjects and normal in one subject. In this small group there was no apparent correlation between osmotic resistance and the number of target cells. The erythrocytes had normal mechanical fragility in both oxygen and carbon dioxide atmosphere.

Erythrocyte survival studies were carried out with cells from each of the 3 subjects in normal individuals. Unexpectedly their survival was markedly and consistently shortened (fig. 6). In one instance, Case 10, the study was repeated in a second normal recipient with essentially the same results. The erythrocyte half-life ranged from six to eighteen days. This behavior of erythrocytes from individuals without demonstrable hemolysis is unique in our experience. Accordingly attempts were made to elucidate the underlying mechanism. The possibility that the rapid elimination of these erythrocytes was the result of an undetected blood group incompatibility seems excluded by the fact that they behaved in the same way in 4 different recipients, none of whom had previously been transfused. Moreover, the presence of unusual antibodies of an immune nature in the sera of the recipients active against the erythrocytes of these subjects was excluded by a negative indirect Coombs test and by cross matching of the serum with trypsin-modified donor cells. The subjects’ own cells invariably gave a negative direct Coombs test. The possibility of an undetected erythrocyte defect dependent on acid hemolysis was ruled out by the appropriate test. Incubation of the patients’ cells in normal plasma, and of normal cells in the patients’ plasma produced no increase in either spontaneous hemolysis or mechanical fragility as compared with normal controls.

**DISCUSSION**

1. *The Combination of Hemoglobin III and Sickle Hemoglobin*

The combination of hemoglobin III with sickle hemoglobin produces a form of sickle cell disease that has not been previously defined. This syndrome would
seem to occupy a position intermediate between the asymptomatic sickle trait and classical sickle cell anemia. It is distinguishable from both these entities on genetic, chemical, hematologic and clinical grounds. The genetic and chemical aspects have already been considered in a previous section.

The clinical-hematologic characterization of the syndrome can be only tentative at the present time. A syndrome consisting of erythrocytic sickling, splenomegaly, and chronic hemolytic anemia would ordinarily be considered sickle cell anemia in the usual sense. On clinical grounds the distinguishing features of our cases were the benign course, the mildness of the anemia and the progressive enlargement of the spleen. We have been unable to find a comprehensive evaluation of the manifestations of sickle cell anemia in children. In our own experience sickle cell anemia in childhood is apt to be considerably more severe, produces a more severe anemia attended by greatly increased, though variable, fecal urobilinogen excretion and commonly leads to cardiac manifestations, musculoskeletal symptoms and impairment in growth and nutrition. The history of 2 of our patients, Cases 2 and 6, indicates that severe crisis-like episodes occurred in their early childhood. In fact, at that time a diagnosis of sickle cell anemia was made. Their subsequent course, however, has been completely asymptomatic over a period of years in contrast to that of sickle cell anemia in which symptoms are apt to recur once the disease has become manifest. Our patients compared favorably with normal children with respect to growth and development. In the 2 older patients the spleen became progressively enlarged despite the asymptomatic course. This is not the expected behavior of sickle cell anemia. In children with this disease the spleen is inconstantly enlarged and tends to become smaller. Marked splenomegaly is usually associated with severe anemia and other manifestations.

Most experienced hematologists have seen cases of mild sickle cell disease in which it is difficult to classify the condition as either sickle cell anemia or the trait. It is probable that some of these cases represent examples of the hemoglobin III-sickle cell combination.* So far it would seem that the syndrome resulting from the combination of hemoglobin III and sickle hemoglobin is relatively benign as compared with typical sickle cell anemia. If further experience confirms these impressions the precise diagnosis of this condition would seem to have important prognostic and eugenic implications.

2. The Combination of the Hemoglobin III and Normal Hemoglobin

The hemoglobin III trait as seen in the individuals whose hemoglobin consists of a mixture of hemoglobin III and normal hemoglobin is an asymptomatic carrier state characterized by target cells and shortened erythrocyte survival. We are aware that the target cell occurs in a wide variety of circumstances. Target cells may be found occasionally in the blood smears of normal individuals. They are markedly increased in sickle cell anemia,26 to a lesser degree in the Mediterranean hemopathies27, 28 and also in the hypochromic anemia of iron

* While this paper was in the process of completion Green and Conley29 called attention to this point. Interestingly enough the hemoglobin of their first patient is said to have an abnormal electrophoretic pattern not identical with the abnormality of sickle cell hemoglobin.
NEW INHERITED ABNORMALITY OF HEMOGLOBIN

deficiency. They may develop in the presence of obstructive jaundice, and following splenectomy or splenic atrophy and may be produced in vitro by exposure of normal erythrocytes to hypertonic media. Even though nonspecific, the presence of many target cells appears to be a significant feature of the hemoglobin III trait. Target cells are greatly increased in the 2 adults and slightly increased in the child. Whether target cells are a constant feature of the trait can only be established by the study of more cases. It is of interest to note that, in a preliminary study of relatives of Family W., numerous target cells were found in a brother of Mr. W., and in 2 of his children. The case reported by Lubitz with an incidence of 67 per cent target cells in a nonanaemic Negro may well represent another example of the hemoglobin III trait.

Target cells which are abnormally thin, are commonly, but not invariably, associated with increased resistance to hypotonic saline, to chemical lysins and to hemolytic sera. The osmotic resistance is increased in 2 of the individuals with the hemoglobin III trait, 1 adult with many target cells and a child with only a slight increase in target cells. It is normal, on the other hand, in 1 adult whose blood also contains many target cells. The literature contains several reports of increased osmotic resistance in normal Negroes, but without reference to the presence or absence of target cells. The question is now pertinent as to whether an unsuspected hemoglobin III trait may account in part for these findings. Elucidation of this problem must await studies on the incidence of the gene for hemoglobin III in the Negro population.

The rapid elimination in normal recipients of erythrocytes from the 3 individuals with the hemoglobin III trait who exhibited no evidence of hemolysis requires comment. Shortened survival of erythrocytes in normal recipients ordinarily bespeaks an intrinsic erythrocyte defect and it is presumed that such defective erythrocytes are also rapidly destroyed in their original host as shown by manifest hemolytic phenomena. The observation that erythrocytes which seem to survive normally in their host are destroyed rapidly in normal individuals appears to be unique.

No immune mechanism could be demonstrated to account for the survival curves. The possibility of a technical error accounting for the survival curves of the erythrocytes of individuals with the hemoglobin III trait appears remote. Erythrocyte survival studies using the method of differential hemagglutination have been performed in our laboratory on a very wide scale for several years. Control survival studies, with normal erythrocytes transfused into normal recipients, yielded the expected normal survival curves during the period of the present investigation. Erythrocytes from one of these individuals were transfused on two separate occasions into different normal recipients with virtually identical results, and similar survival curves were obtained when erythrocytes from the other 2 individuals were transfused into normal recipients. Similarly shortened survival times were obtained with erythrocytes from patients with a mixture of hemoglobin III and sickle hemoglobin. These individuals, with respect to the inheritance of a single sickling gene, are comparable to the sickle trait which, by itself, is not associated with shortened erythrocyte survival. The rapid elimination of their cells may be ascribed either to the hemoglobin III per se, or to interaction between the two hemoglobins.
The conclusion seems inescapable that the shortened survival of erythrocytes containing hemoglobin III and normal hemoglobin reflects a hereditary intracorpuscular erythrocyte defect and that in the affected individuals there exists a protective mechanism which is lacking in normal individuals. At present the nature of this mechanism is totally obscure.

Measurements of the pH of the blood of 1 individual, Case 5, revealed no deviation from the normal. In vitro experiments in which normal erythrocytes were incubated with serum of these individuals, and vice versa, yielded no significant information. Further studies are now in progress to elucidate this phenomenon and to establish whether it is of constant occurrence.

3. Relationship of Hemoglobin III to Mediterranean Disease

Target cell syndromes resembling Mediterranean anemia have been described in the Negro, and sickle cell disease has been reported in Caucasians, chiefly of Mediterranean ancestry. Moreover, a new variant of sickle cell disease resulting from the interaction of the sickling gene and the thalassemia gene has already been identified. It is therefore pertinent to examine the relationship of hemoglobin III to Mediterranean disease.

The combination of the sickling and thalassemia genes was described in families of Italian and Sicilian ancestry by Silvestroni and Bianco, and more recently in this country by Powell, Rodarte, and Neel. Although clinically resembling sickle cell anemia the condition appears to be distinguished hematologically from that entity by the presence, apart from sickling, of hypochromasia and microcytosis. In these families those parents who contributed the thalassemia gene were clearly identifiable as carriers of the Mediterranean trait by their hematologic features. Some of the siblings, and, in one instance, children of the affected individuals likewise presented the picture of the Mediterranean trait.

In view of the similarity of the interaction between the thalassemia and sickle cell genes, and the hemoglobin III and sickle cell genes, the possibility must be considered that the so-called hemoglobin III is actually the abnormal hemoglobin found in thalassemia, modified by its introduction into a somewhat different genetic background. This possibility deserves all the more careful examination because of the recent discovery by Singer and his associates and independent unpublished observations from our laboratory (Baylor) that an abnormal hemoglobin component may be demonstrated in thalassemia by the method of fractional alkaline denaturation. With this method preliminary studies indicate a similarity between hemoglobin III and thalassemia hemoglobin. As yet we have no information on the electrophoretic pattern of hemoglobin in Mediterranean disease, but on hematologic grounds it does not appear that hemoglobin III and Mediterranean hemoglobin are identical.

Those of our patients who had the combination of hemoglobin III and sickle cell hemoglobin (Cases 1, 2 and 6) did not show the hypochromic, microcytic blood picture emphasized by Silvestroni and Bianco for the combination of thalassemia and sickle cell trait. Even more clear-cut differences are apparent when the subjects with a mixture of hemoglobin III and normal hemoglobin are compared with carriers of the Mediterranean trait. The two groups are comparable on genetic grounds since each represent heterozygotes carrying one gene...
for the abnormal trait and its normal allele. Except for the presence of target cells there are no similarities between the two groups. The hemoglobin III trait lacks the microcytosis, ovalocytosis, and polycythemia characteristic of the Mediterranean carrier state. Moreover, the strikingly rapid elimination of transfused erythrocytes from patients with the hemoglobin III trait is in marked contrast to the essentially normal mode of elimination of Mediterranean trait cells.45, 46

While the available evidence seems to indicate that the condition described by us is not identical with the Mediterranean syndromes the possibility remains that conversely some of the cases reported as Mediterranean disease in the Negro were actually related to the presence of hemoglobin III. In an exhaustive study Schwartz and Mason38 reported Mediterranean anemia in 4 Negroes and presented considerable supportive evidence covering their families. From the description of the patients themselves it would seem that the cases conformed to the typical picture of Mediterranean anemia, rather than to that described by us. In several members of the families, however, Schwartz and Mason observed target cells in far greater number than seem to be characteristic of the Mediterranean trait. They reported 50 to 100 per cent target cells in several of these individuals whereas Dameshek found only 1 to 8 per cent in individuals with the Mediterranean trait,27 figures which are in keeping with our own experience. The high incidence of target cells in the relatives of Schwartz and Mason’s patients, in the absence of erythrocyte sickling, is comparable to the findings in our cases. The possibility cannot be excluded that some of the cases reported as Mediterranean hemopathies in the Negro were, in reality, examples of the hemoglobin III trait, or even the as yet unrecognized homozygous state.

Further experience with a larger number of cases exhibiting the hemoglobin III trait may disclose a wider spectrum of clinical or hematologic features and may well show some degree of overlapping in the manifestations of this syndrome and the Mediterranean hemopathies. The ultimate separation of these syndromes will rest upon the identification of hemoglobin III by chemical, or perhaps immunochemical means.

SUMMARY

Through clinical, genetic and physicochemical studies a new inherited abnormality of hemoglobin has been recognized in American Negroes. The new hemoglobin, provisionally called hemoglobin III, can be separated from both normal and sickle cell hemoglobin by electrophoretic analysis. The structural anomaly of the hemoglobin molecule is determined by a gene inherited as a simple Mendelian dominant.

A distinct hemolytic syndrome which is intermediate between the benign sickle cell trait and sickle cell anemia results from the combination of the new hemoglobin with sickle cell hemoglobin. A tentative characterization of the syndrome is presented. In contrast to classical sickle cell anemia this form of sickle cell disease is characterized by a mild hemolytic anemia with slowly progressive splenomegaly in the absence of cardiac or musculo-skeletal manifestations. In vitro, the erythrocytes sickle like those of sickle cell anemia. The bone marrow shows erythroid hyperplasia, fecal urobilinogen excretion is increased, and the
survival time of the erythrocytes in normal recipients is shortened, but in the patients the red cell count and hemoglobin concentration are only slightly depressed. Reticulocytosis is slight and icterus is not observed. Whereas in sickle cell anemia both parents are expected to have the sickle trait, only one parent of these individuals shows sickling, while the nonsickling parent is a carrier of the hemoglobin III. The new syndrome does not appear to be identical with that resulting from the simultaneous presence of the sickling gene and the thalassemia gene.

The presence of hemoglobin III when combined with structurally normal hemoglobin is expressed as an asymptomatic carrier state. The erythrocytes do not sickle but have a high incidence of target cell deformity and increased resistance to hypotonic saline. Although there is no evidence of hemolysis in such individuals their erythrocytes are eliminated with abnormal rapidity from the circulation of normal recipients.

The homozygous state with respect to this new hemoglobin has not yet been identified but may well be some already recognized atypical form of chronic hemolytic anemia.

Studies are now in progress to determine the incidence of this new molecular abnormality of hemoglobin.

REFERENCES

1258

NEW INHERITED ABNORMALITY OF HEMOGLOBIN


A New Inherited Abnormality of Hemoglobin and Its Interaction with Sickle Cell Hemoglobin

EUGENE KAPLAN, WOLF W. ZUELZER and JAMES V. NEEL