Paroxysmal Nocturnal Hemoglobinuria with Elevated Fetal Hemoglobin

By Anne A. Rassiga-Pidot, Gibbons G. Cornwell, III, and O. Ross McIntyre

A persistent elevation of the fetal hemoglobin (Hgb F) level (5%-15%) was observed in a 22-yr-old white male with paroxysmal nocturnal hemoglobinuria (PNH). Acid treatment of the peripheral smear (Betke-Kleihauer technique) demonstrated a heterogeneous distribution of Hgb F in the red cells. As expected, the lowest acetylcholinesterase activity and the most hemolysis after acid stress were found in the low-density, reticulocyte-rich cell fraction. In contrast, the highest Hgb F levels were found in the high-density, reticulocyte-poor fraction. Further evidence for the segregation of these two abnormalities was obtained from the observation that less Hgb F was present in the hemolysate obtained after acid lysis than was present in the mixed blood sample or the remaining nonhemolyzed red cells.

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by acute and chronic intravascular hemolysis, pancytopenia, and episodes of thrombosis. PNH red cells lyse in an acid environment and when reacted with antibody and complement. Both mechanisms involve complement activation probably via the alternative C3 activator system. Reticulocyte-rich subpopulations of red cells contain a larger proportion of these complement sensitive cells which have a shortened in vivo survival. A decreased concentration of the red cell membrane enzyme acetylcholinesterase (AChE) is also found, and the red cell fractions which are most deficient in AChE are the most sensitive to complement-mediated lysis.

During investigation of a nonanemic patient with a mild form of PNH, a persistently high level of fetal hemoglobin (Hgb F) was found which was distributed among the red cells in a heterogeneous fashion. The present study was undertaken to identify the red cell population with the PNH defect and elevated Hgb F, and to correlate these abnormalities. We found that the population of red cells containing the highest proportion of cells lysed in acid was distinct from that containing the highest concentration of Hgb F.

CASE REPORT

J.C., a 21-yr-old college senior, presented to the Dartmouth College Student Health Service in January 1971 with a history of abrupt onset of red urine. In 1965 he had an episode of hematuria following severe physical exercise. An intravenous pyelogram was normal. Subsequently he has had two or three episodes of red urine yearly and in 1969 was found to have free hemoglobin in his

From the Department of Internal Medicine, Dartmouth-Hitchcock Medical Center, Hanover, N. H. 03755.


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Anne A. Rassiga-Pidot, M.D.: Instructor, Department of Medicine, Dartmouth Medical School, Hanover, N.H. 03755: Presently Assistant Chief, Hematology Section, VA Hospital and Assistant Professor, Department of Medicine, Case Western University Medical School, Cleveland, Ohio 44106: Recipient of a Postdoctoral Fellowship 5-F03-CA42989 from the NCI. Gibbons G. Cornwell, III, M.D.: Assistant Professor, Department of Medicine, Dartmouth Medical School, Hanover, N.H. 03755. O. Ross McIntyre, M.D.: Associate Professor, Department of Medicine, Dartmouth Medical School, Hanover, N.H. 03755.

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Table 1. Hgb F Levels in Three Patients With PNH

<table>
<thead>
<tr>
<th>Patient</th>
<th>Whole Blood</th>
<th>Top 2%</th>
<th>Bottom 2%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hgb (g/100 ml)</td>
<td>Hgb F (%)</td>
<td>Retic (%)</td>
</tr>
<tr>
<td>J.C. (2/19/71)</td>
<td>14.1</td>
<td>15.0</td>
<td>4.3</td>
</tr>
<tr>
<td>J.C. (3/1/71)</td>
<td>14.9</td>
<td>11.5</td>
<td>3.2</td>
</tr>
<tr>
<td>J.C. (6/19/71)</td>
<td>15.1</td>
<td>13.5</td>
<td>0.9</td>
</tr>
<tr>
<td>J.C. (9/8/71)</td>
<td>14.3</td>
<td>5.2</td>
<td>1.5</td>
</tr>
<tr>
<td>J.C. (9/13/71)</td>
<td>14.0</td>
<td>7.1</td>
<td>1.5</td>
</tr>
<tr>
<td>R.D. (5/12/71)</td>
<td>8.1</td>
<td>0</td>
<td>9.5</td>
</tr>
<tr>
<td>H.T. (5/26/71)</td>
<td>12.3</td>
<td>0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

urine and an elevated Hgb F level (11.6%). The present episode occurred spontaneously. There was no other significant past medical or family history. Physical examination was normal.

The urine was dark red and reacted positively with benzidine but did not contain red cells. Hemosiderin was present in the sediment. The remainder of the urine examination, including culture, was normal. The Hgb was 13.7 g/100 ml, Hct 38%, WBC 4300/cu mm with 74 polymorphonuclear leukocytes, 20 lymphocytes, 5 monocytes, and 1 eosinophil; the platelet count was 120,000/cu mm, and the reticulocyte count was 4.4%. Red cell indices were normal. The peripheral smear showed rare spherocytes. Five subsequent blood counts obtained over an 8-mo period showed similar findings. The plasma free hemoglobin level was 14.6 mg/100 ml and 39 mg/100 ml on two occasions (normal 1-4 mg/100 ml). Other laboratory data included normal serum bilirubin and depressed haptoglobin and hemopexin levels. A direct Coombs test was negative. G6PD screen and Heinz Body preparations were normal. The leukocyte alkaline phosphatase score was 28 (normal 21-100). Sucrose hemolysis and acid hemolysis tests were positive. Hemoglobin electrophoresis on cellulose acetate suggested an increase in Hgb F which was confirmed by quantitation (Table 1). The Hgb A2 level was 1.7% (normal 1.5%-3.3%). The patient’s red cell membranes did not contain complement fractions C3 or C4 (kindly determined by Dr. Harvey Colton, Harvard Medical School).

Blood studies performed on the patient’s mother and father showed normal values for Hgb, WBC, platelets, reticulocyte count, and fetal Hgb levels. The Hgb electrophoreses were normal, and the sugar water tests were negative. Two siblings were not available for study. The ABO and Rh blood groups of the parents and the patient were compatible.

The patient has done well on no specific therapy, maintaining a normal hemoglobin despite strenuous physical activity.

MATERIALS AND METHODS

The presence of fetal hemoglobin in individual red cells was determined by the Shepard modification of the acid elution method of Kleihauer and Betke. Hgb F was quantitated by the alkali denaturation method of Jonxis and Visser, and the results are expressed as a per cent of the total hemoglobin. Control experiments showed that the acid treatment of certain blood samples prior to measurement of fetal hemoglobin by this method did not nonspecifically alter the Hgb F level provided sufficient alkali was used. At dilute hemoglobin concentrations, however, this method was not accurate. Consequently, when the Hgb F was measured in the supernatant obtained after acid hemolysis of sensitive cells, an increased volume of sample was required. The appropriate pH was achieved either by adding excess NaOH or by dialysis to remove the acid. Acetylcholinesterase levels in red cell membranes were measured by the spectrophotometric method of Ellman et al.

Quantitation of the acid and sucrose hemolysis was obtained by the standard benzidine technique.

The red cells were fractionated into young and old populations by a modification of the ultracentrifugation method of Riss. Blood samples, drawn into ACD solution (1 ml ACD to 5 ml blood), were centrifuged at 1000 g for 10 min to remove plasma. The red cells were then spun in a Beckman SW 27 rotor at 131,000 g for 1 hr at 4°C.
RESULTS

Quantitative Hgb F Determination

The percentage of Hgb F present in the blood of patient J.C. was found to vary between 5.2% and 15% over a 7-mo period (Table 1). No correlation was noted between the hemoglobin concentration, the reticulocyte count, the percentage of cells hemolyzing on exposure to acid, and the Hgb F level. The patient was never anemic. Two other patients with PNH (R.D., H.T.) were not found to have elevated Hgb F levels despite significant anemia.

After centrifugation of J.C.’s red cells, a decreased quantity of Hgb F was found in the young, reticulocyte-rich, top 2% fraction in comparison to whole blood or to the bottom fraction (Table 1).

Blood samples obtained from J.C.’s parents contained no Hgb F.

Red Cell Distribution of Hgb F

The Hgb F was distributed in a heterogeneous fashion in the red cells of J.C. A similar distribution of Hgb F was noted in the cells remaining after exposure to acid. Only occasional cells containing small amounts of Hgb F were observed in smears obtained from the parents of J.C., a normal finding.

Acid and Sucrose Hemolysis Tests

Red cells from J.C. hemolyzed when incubated with acid in the presence of fresh isologous serum (Table 2), but did not hemolyze in inactivated serum (56°C for 1 hr). The per cent hemolysis after acid exposure was 7.9%, 6.6%, and 17.2% on three occasions. Acid hemolysis tests performed on patients R.D. and H.T. showed 20.6% and 7.2% hemolysis, respectively.

After fractionation of red cells, lysis after exposure to acid was found to be greater for the young cells (33.1%) than the old cells (14.7%) (Table 2).

Quantitative acid hemolysis tests performed on whole blood and red cell fractions of two normal individuals revealed no increased acid sensitivity in any fraction, indicating that the process of centrifugation did not damage the cell nonspecifically.

Acetylcholinesterase Levels of Red Cells

The mean level of red cell AChE for 12 normal subjects was $1.16 \times 10^{-14}$ moles/min/RBC. J.C. and the two other patients with PNH fell within the normal range. However, unlike the normal subjects, or two patients with hemolysis for other reasons, the patients with PNH had low AChE in their young red cells (top 2%), despite reticulocyte counts in those fractions of 13%–86% (Fig. 1).

<table>
<thead>
<tr>
<th>Table 2. Per Cent Hemolysis and the Distribution of Hgb F in the Fractions of J.C.’s Red Cells After Acid Stress</th>
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</thead>
<tbody>
<tr>
<td>Blood Sample</td>
</tr>
<tr>
<td>Whole Blood</td>
</tr>
<tr>
<td>Top 2%</td>
</tr>
<tr>
<td>Middle 2%</td>
</tr>
<tr>
<td>Bottom 2%</td>
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</table>
Acid Sensitivity of Cells Containing Hgb F

Following the acid hemolysis reaction, quantitative Hgb F determinations were carried out on the mixed sample, red cells alone, and the supernatant fluid. It was found that the percentage of Hgb F present in the supernatant was lower than that of the mixed sample or the remaining red cells (Table 2). Similar results were found in all the red cell fractions obtained after centrifugation.

DISCUSSION

The clinical and laboratory features of the illness of J.C. satisfy the standard criteria for the diagnosis of PNH. The reticulocyte-rich red cell fraction of J.C.'s blood contained more cells which hemolyzed in an acid environment than the reticulocyte-poor fraction, a finding similar to the data of others. In addition, although a normal AChE level was found when whole blood was studied, a low AChE level was obtained in the reticulocyte-rich fraction in contrast to the high levels of AChE found in normal reticulocytes.

The unique finding in our patient was the persistent elevation of Hgb F which comprised 5.2%–15% of his total circulating hemoglobin concentration and was distributed unevenly among his red cells. Elevation of Hgb F has not previously been reported in PNH, although mild elevations of Hgb F have been observed in some anemic PNH patients (David Jenkins, personal communication).

We initially hypothesized that the increased production of Hgb F was a second manifestation of a red cell abnormality and that the majority of the Hgb F would be found...
in that population of red cells most severely affected with the membrane defect of PNH. Our data, however, does not support this hypothesis. After centrifugation, the highest levels of Hgb F were found in the reticulocyte-poor red cell fraction. In addition, the hemoglobin released into the supernatant after an acid stress contained proportionally less Hgb F than that present in the whole sample, irrespective of red cell age. We conclude, therefore, that the population of cells containing Hgb F is not the same as the population which hemolyzes most readily in response to acid and may represent a population which lives longer in vivo. This situation is analogous to that seen in β-thalassemia.14-16 It should be noted, however, that a complete segregation of the defects was not observed, since the supernatant after acid hemolysis contained some Hgb F.

The stimulus for the production of Hgb F in hematologic disease is not known. Although elevated Hgb F levels are often associated with diseases which cause anemia, anemia or hypoxemia alone does not result in increased Hgb F synthesis.17 In anemic conditions associated with increased Hgb F levels, correction of anemia does not have a predictable effect on Hgb F synthesis.18-20 In certain diseases, it is probable that Hgb F synthesis is a nonspecific compensatory response to an abnormality of the erythron. In β-thalassemia, for example, it is presumed that the production of γ chains has a protective effect on the cell by preventing the aggregation and precipitation of free α chains.

Elevated Hgb F levels in individuals beyond early childhood have been reported in three major situations: (1) as an isolated finding in hereditary persistence of Hgb F, (2) associated with disorders of hemoglobin synthesis or hemolytic anemias,17,21-23 and (3) associated with primary bone marrow disease, such as aplastic anemia, pernicious anemia, multiple myeloma, leukemia, and myelofibrosis.17-19,22-24 The normal RBC morphology of J.C. and his parents and the absence of elevated Hgb F levels in J.C.’s parents make it unlikely that any of the conditions in group 1 or 2 explain the elevated Hgb F in our patient. In the 3rd group, Hgb F is distributed heterogeneously throughout the cells. Although moderate to severe anemia is often present in these conditions, some patients without anemia have been reported.18,19

The patient under discussion has a disorder (PNH) which is felt to be acquired and may represent a preleukemic condition.25 He falls most likely, therefore, into the 3rd group of disorders characterized by elevated Hgb F. Although it is not clear what mechanisms are responsible for the defects in this patient’s red cells, it appears that the two abnormalities are neither confined to the same clone of red cells nor expressed completely independently. These findings imply a pattern of mosaicism in the marrow population.

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