Paroxysmal Nocturnal Hemoglobinuria: Evidence for Monoclonal Origin of Abnormal Red Cells

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Paroxysmal nocturnal hemoglobinuria (PNH) was diagnosed in a 26-year-old Nigerian woman who subsequently died of amebic colitis. The patient's red cells exhibited mosaicism with respect to glucose 6-phosphate dehydrogenase, in that some of them had the A variant of this enzyme (as expected in female subjects heterozygous at this sex-linked locus). The red cells bearing the PNH abnormality only had the B variant, suggesting that they all belonged to a single abnormal clone.

In contrast to the other hemolytic disorders associated with an intrinsic defect of the erythrocyte, paroxysmal nocturnal hemoglobinuria (PNH) is not a genetically determined disease (evidence reviewed by Crosby; Dacie). Accordingly, it has been suggested that the erythrocyte population with the PNH abnormality may consist of a clone of cells which arose by somatic mutation. Here we present experimental support for this idea. We found that in a patient suffering from PNH, and who exhibited erythrocyte mosaicism with respect to glucose 6-phosphate dehydrogenase, all the red cells carrying the PNH abnormality were homogeneous with respect to that marker.

The data were obtained while studying a patient with PNH (a condition not yet described in tropical Africa), who also illustrates an exceptional complication of the disease, unfortunately fatal in our case: fulminating amebic colitis.

Methods

Routine hematologic investigations were carried out by standard technics. The acidified serum test and the test for the Donath-Landsteiner antibody were carried out as described by Dacie and Lewis.

Electrophoresis of glucose 6-phosphate dehydrogenase. Blood was collected in one fifth volume of ACD solution and the red cells washed twice by centrifugation in normal saline (containing 0.1 mM EDTA). The sedimented cells were hemolyzed in 10 mM NADP and the hemolysates subjected to starch gel electrophoresis as previously described.

Case Report

Mrs. F.A., a 26-year-old Yoruba seamstress, was admitted to Adeoyo State Hospital on September 30, 1968, with a complaint of "passing dark urine, like blood," which had...
started in the night five days previously, and was associated with diffuse abdominal pain, weakness, dizziness and effort dyspnea. She denied having taken any drug at the time. She was five months pregnant, and had had two previous pregnancies, during which she had been treated for "anemia and weakness." Physical examination revealed marked pallor of palmar skin and of mucous membranes in a young woman who was otherwise in good general condition. The patient was very weak, but afebrile and in no acute distress. Laboratory investigations revealed a hemoglobin of 5.3 Gm./100 ml., with a hematocrit of 19 per cent and a mean corpuscular hemoglobin concentration of 29 per cent. The WBC count was 3400 with 61 per cent polymorphs, two per cent eosinophils, 35 per cent lymphocytes, and two per cent monocytes. The blood film showed marked hypochromia, anisocytosis and polychromasia; no malaria parasites were present. Hemoglobin electrophoresis showed only hemoglobin A. The urine gave a positive test for protein, but did not contain, on admission, hemoglobin or urobilinogen. The sediment contained few red cells and few white cells. The blood group was B, Rhesus positive and the direct Coombs test was negative.

The provisional diagnosis was of severe anemia in pregnancy, a condition very common in Nigeria. She was treated with antimalarials, oral and parenteral iron and folic acid.

Progress

On the night of the sixth hospital day the patient developed a fever of 103°F and passed red colored urine. The following morning she looked more anemic and had developed slight jaundice. She complained of lower abdominal pain, vomited once, and continued to pass dark urine. At this time her hematocrit was 15 per cent. The WBC was 2400 with a normal differential, the reticulocyte count was five per cent and the platelet count 160,000. The plasma hemoglobin concentration was 230 mg./100 ml., and the urine also contained free hemoglobin, as confirmed by spectroscopy. The level of erythrocyte glucose 6-phosphate dehydrogenase was normal, and the test for the Donath–Landsteiner antibody was negative. The acidified serum test was positive on two successive occasions (the percentage of cells lysed was 23 and 28 respectively), which led to a diagnosis of PNH. The patient was transfused with 750 ml. of packed red cells. She had further episodes of hemoglobinuria at irregular intervals; she required one additional transfusion and was also placed on penicillin and streptomycin, and later on tetracycline (Ledermycin). However, the patient continued to be pyrexic and developed a gluteal abscess. On the 16th hospital day she went into premature labor and delivered a live male baby which weighed 750 Gm. and died few minutes after. Blood loss at delivery was estimated at 100 ml. After her miscarriage the patient improved slightly, but the temperature remained high and was now remittent. Her appetite was poor but fluid intake was adequate. On the 20th night in hospital the patient developed signs and symptoms of acute abdominal catastrophe (mesenteric thrombosis?) and died before surgery could be undertaken.

Autopsy Findings

All serous cavities contained bile-stained fluid (pericardial, 25 ml.; right pleural 100 ml.; left pleural 55 ml.; peritoneal 1350 ml.). The lungs showed congestion and severe edema. The heart chambers were dilated and the myocardium was flabby and pale. The kidneys weighed 190 Gm. each: they were markedly enlarged and soft in consistency. The cortical surface had a dirty brown discoloration and scattered whitish foci. The cut surface showed blurring of the corticomedullary junction, calices and pelvis. Histologically, some of the glomeruli were hyalinized, and there was some atrophy of the convoluted tubules. Prussian blue stain showed very heavy iron deposition, which was especially marked in the epithelial cells of the proximal tubules and Henle’s loops. A few of the glomeruli were also involved. The uterus showed evidence of recent parturition and involution: it contained a small quantity of septic hemorrhagic exudate. In the fundus of the stomach, there was an acute hemorrhagic ulcer involving all layers of the gastric wall. In the small intestine, the Peyer’s patches were prominent but not ulcerated. In the terminal ileum there was a single transverse necrotic ulcer. The cecum, the ascending colon, the transverse
Fig. 1.—Starch gel electrophoretic analysis of glucose 6-phosphate dehydrogenase from patient's whole hemolysate and from cells with PNH abnormality. To obtain enzyme from latter cells, acidified serum test was carried out on 0.5 ml. of patient's red cells. At end of one-hour incubation, 23 per cent of cells had lysed. Remaining cells eliminated by centrifugation, the supernatant concentrated 10-fold by dialysis against solid Aquacide (California Biochemicals), and glucose 6-phosphate dehydrogenase from lysed cells, analyzed by starch gel electrophoresis. Origin (not shown) below bottom of figure, and migration toward anode (top). (1) Glucose 6-phosphate dehydrogenase type A, control; (2) type B, control; (3) patient's whole hemolysate; (4) patient's PNH cells.

colon and the descending colon were the seat of severe acute colitis with extensive confluent ulcers. The ulcers were covered by necrotic purulent exudate and had undermined edges; they involved the whole thickness of the bowel, with associated localized peritonitis. Histologically, both the superficial and the undermined type of ulcers were seen, with numerous amebae in the mucosa and submucosa. There was edema and marked sloughing of the mucosa, as seen in very acute cases, but no cellular reaction. The intervening mucosa between the ulcerated areas was unaffected. The liver was enlarged (2210 Gm.), but had normal consistency. Cut surfaces showed mild chronic venous congestion and diffuse fatty change. The spleen (175 Gm.) showed small subcapsular areas of hemorrhagic necrosis. The right femoral bone marrow was very pale and contained abundant fat. Histologically, between the fat spaces myeloid and erythroid elements were fairly well represented, in a ratio of approximately 1:1. There was no fibrosis. In the central nervous system, the cerebrospinal fluid was bile stained. All other organs did not show significant abnormalities.


ANALYSIS OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE IN PATIENT'S RED CELLS

Female subjects who are heterozygous at the glucose 6-phosphate dehydrogenase locus are known to exhibit red cell mosaicism with respect to this enzyme. This was the case in our patient, who had a phenotype A-B (for nomenclature, see WHO) on starch gel electrophoresis (Fig. 1), meaning that some of her red cells had the A type and some had the B type of glucose 6-phosphate dehydrogenase. However, the erythrocytes that were susceptible
to lysis in the acidified serum test only had the B type of this enzyme (see Fig. 1, and Discussion).

Several possible artefactual causes for the difference in glucose 6-phosphate dehydrogenase type between whole patient's cells and PNH cells were considered and eliminated. The A-B phenotype of the patient was the expression of genuine heterozygosity, because it was detected on a sample of blood obtained before transfusion. The handling of enzyme released from the PNH cells in the course of acid hemolysis does not affect its electrophoretic mobility. This was shown by adding acidified serum, concentrated tenfold, to authentic samples of glucose 6-phosphate dehydrogenase type A and type B. A similar check was done on the red cells from another (male) patient, who had glucose 6-phosphate dehydrogenase type A, and also carried the PNH abnormality. The enzyme from the abnormal cells, obtained by lysis in acidified serum as described in the legend to Fig. 1, still behaved electrophoretically as A.

**DISCUSSION**

**Clinical Features of Case Reported**

The patient described had the full-blown clinical and hematologic picture of paroxysmal nocturnal hemoglobinuria. In addition to the cardinal symptom that names the disease, she had anemia which was exacerbated by pregnancies, a hypoplastic marrow, evidence of secondary iron deficiency, a positive acidified serum test and an excessive proneness to acute infections.

Pregnancy is rare in patients with PNH and miscarriage is often the outcome. Amoebic colitis tends to be specially severe in pregnancy and puerperium. In our patient the unique association of PNH, pregnancy and amoebiasis probably helped the infection to become fulminating. The bowel ulcerations revealed at autopsy appeared to be less than one week old. (This may explain the absence of diarrhea and the mistaken assumption that the patient's abdominal symptoms were due to mesenteric occlusion, a rather common complication of PNH.)

**PNH in Nigeria**

This seems to be the first case of PNH reported from tropical Africa. We were able to find in the literature only three cases from South Africa and some cases in Americans of African descent. However, since this is a rare disease everywhere, and many cases may remain undetected for a number of reasons, it is possible that its frequency in Nigeria is no less than in countries outside Africa.

**Origin of Abnormal Cells in PNH**

Various lines of evidence indicate that two different red cell populations exist in patients with PNH: (1) In vitro, the proportion of cells that are lysed by acidified serum (or by antibody and complement) varies from one patient to another, but tends to be constant in the individual patient. When sera

*Dr. Elizabeth Topley (personal communication) found a transient weekly positive acidified serum test in a 25-year-old Nigerian man with aplastic anemia.*
from different donors are used in the test, or successive additions of the same serum are made, until maximal hemolysis is reached, a fraction of the patient's cells are left untouched, and this fraction is characteristic of the individual patient.2,23 (2) In vivo, survival curves of the patient's own erythrocytes often exhibit two components, one corresponding to cells with a normal life span, and one corresponding to cells with a markedly reduced lifespan.24 (3) Clinical severity in different patients is affected by various factors,23 but it correlates roughly with the percentage of red cells susceptible to lysis in the acidified serum test, suggesting that the extent of hemolysis in vivo depends on the proportion of the patient's cells carrying the PNH abnormality.20

As to the mechanism whereby the abnormal cells arise, two different models appear possible in principle: (1) All the patient's erythroid cells are originally normal: but after they are produced, a random fraction of them acquires the peculiar susceptibility to lysis which is characteristic of PNH. (2) The patient produces two kinds of erythroid cells: normal cells, and "PNH cells," the latter constituting a clone arisen by somatic mutation. The "abnormal clone" hypothesis is presently favored by most authors.2,25

The second model predicts that the PNH erythrocytes will be homogeneous with respect to all genetic markers, including those for which mosaicism is found in red cells. One such marker is glucose 6-phosphate dehydrogenase, which has already been used in testing the monoclonal versus polyclonal origin of various tumors.26-28 In Nigeria, there are three common alleles for glucose 6-phosphate dehydrogenase (A, B and A'); for nomenclature see Ref. 12), and on the basis of the known gene frequencies29 most female subjects are expected to be heterozygous at this sex-linked locus. It is known that, as originally proposed by Lyon30 for other sex-linked characters, heterozygosity at the glucose 6-phosphate dehydrogenase locus entails mosaicism for this enzyme in somatic cells.31,32 For instance, in our patient, who had the A-B phenotype (see Fig. 1) (and by inference the GdA/GdB genotype; see Ref. 12), about half the red cells have the A enzyme and about half the B enzyme. However, her "PNH cells" only had the B type (Fig. 1). We interpret this finding as indicating that, at least in this patient, PNH cells were monoclonal in origin. The abnormal clone has presumably arisen by a single somatic mutational event that occurred in one of the cells in which the A gene for glucose 6-phosphate dehydrogenase happened to be on the inactive X-chromosome, and the B gene on the active one.

Although we consider that the data presented, in conjunction with those discussed by others2-4,25 are strongly in favor of the single-clone hypothesis, the following points will require clarification in future:

*We have recently studied another female patient with PNH, whose erythrocyte glucose 6-phosphate dehydrogenase activity exhibits "intermediate deficiency" and is electrophoretically of type A (presumed heterozygote genotype: GdA/GdA'; see Ref. 12). When the enzyme activity of her red cells was measured and expressed in units/Gm. of hemoglobin12 the following values were obtained—whole population: 4.3; PNH cells: 1.1; cells unlysed in the acid hemolysis test: 7.7. Thus, again the PNH cells do not seem to represent a random sample of the whole erythrocyte population, but rather a homogeneous fraction having, in this case, the deficient rather than the normal enzyme.
We cannot rule out the possibility that in our experiment erythrocytes with the A type of glucose 6-phosphate dehydrogenase were not represented amongst the PNH cells because they had been selectively destroyed in vivo. For this to occur, one would have to make the unlikely ad hoc hypothesis that PNH cells have a different susceptibility to in vivo hemolysis according to which variant of glucose 6-phosphate dehydrogenase they contain.

It is unlikely that some (type A) PNH cells were not detected because they are selectively resistant to in vitro hemolysis. It is possible that a larger number of cells might have lysed if we had employed, for instance, the "sucrose test"33 or the "cold antibody hemolysis test"34 instead of the acid hemolysis test. But we cannot visualize a mechanism whereby, in any system, type-A cells would be selectively spared, if these were represented among the PNH cells.

If the model of an abnormal clone in accepted, one can try to visualize the pathogenesis of the disease as follows. (1) A somatic mutation takes place in a "stem cell" (presumably multipotent, since abnormalities are detected not only in erythroid cells, but also in myeloid cells and platelets; see Ref. 2). (2) If there are \( n \) stem cells in the body, the clone of mutated cells could never amount to more than \( 1/n \) of the total erythroid cell population, unless the mutation entails some selective advantage for the cell, e.g., in the rate of proliferation. (3) If the mutation does carry a selective advantage, the size of the clone will gradually increase† to more than \( 1/n \) of the total erythroid cell population, and will eventually comprise a sizable portion thereof, when PNH will become detectable. (4) The time at which this happens will depend on the time at which the mutation has occurred and the ratio in proliferation rate between the abnormal and the normal cells. Thus, it is theoretically possible that the mutation has taken place very early in life even in patients who present in the typical age group (third to fourth decade). In our case, the evidence indicates only that the abnormal clone arose after fixation of the active versus inactive X-chromosome (which takes place very early in embryonic life5,35), but it does not indicate how long after. The rare cases of PNH diagnosed in childhood,139,41 may represent extreme instances, in which the mutation has occurred very early, or the rate of proliferation of the abnormal clone is unusually high. At the other extreme, the rare cases of "cured" PNH41 may be explained if the abnormal clone is in turn outgrown by the normal cells, perhaps as a result of a new mutational event.

At first sight one would expect that the tendency of PNH cells to lyse will be a selective factor against them (in which case the abnormal clone could never establish itself). It is likely that only mature red cells are involved in premature destruction, while production of new abnormal cells may be faster than production of normal cells. More PNH cells are found among reticulocytes than among erythrocytes.33-37

†The fact that the percentage of PNH cells tends to be roughly constant in a given patient15,26 is not in conflict with the postulated increasing size of the abnormal clone, for the following reasons: the tests are usually carried out only well after the disease is already established, and therefore most of the "growth" of the clone has already taken place; and the tests only measure the percentage of PNH cells in circulating blood, which will be greatly influenced by the variable rate of in vivo destruction of the abnormal cells. A method of measuring the percentage of PNH cells as they are produced is not yet available.
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