Two Populations of Erythroid Cell Progenitors in Paroxysmal Nocturnal Hemoglobinuria

By Bruno Rotoli, Renato Robledo, Nicola Scarpato, and Lucio Luzzatto

We have grown erythroid cell colonies from two patients with paroxysmal nocturnal hemoglobinuria (PNH). At 11 to 13 days, individual bursts were picked and incubated for 24 hours with ²H-leucine in order to label total cell protein (mainly hemoglobin). After appropriate washing, each burst was subjected to a miniaturized acidified serum test, and lysis was measured by the release of radioactivity. In bursts from normal controls, lysis was 19% ± 13% SD. By contrast, of 58 bursts from PNH patients, 14 had lysis similar to that of controls (mean 15.4% ± 10.6%), while 44 had lysis ranging from 42.2% to 85.8% (mean 70.3% ± 10.4%). Colonies sensitive to acidified serum were acetylcholinesterase (AchE) negative, whereas normal colonies were AchE-positive. Thus, based on two independent criteria, a dual population of erythroid burst-forming units (BFU-E) can be demonstrated in PNH. These data confirm directly the somatic mutation model of the pathogenesis of PNH, and by these methods the relative sizes of the normal and the PNH cell populations can be measured at the level of the erythroid cell precursors.

PAROXYSMAL NOCTURNAL hemoglobinuria (PNH) is a chronic hemolytic disorder defined and identified by the acidified serum test. It is well known that, in this test, only a fraction of the patient’s red cells are lysed by virtue of their hypersensitivity to activated complement, with the remaining cells being not susceptible to lysis. On this basis, Dacie suggested in 1962 that there are two distinct red cell populations in the patients’ blood that can be referred to as “normal” and “PNH.” This suggestion was corroborated by the findings of (a) biphase ⁵¹Cr-labeled red cell survival curves and (b) biphase (and sometimes triphasic) cold antibody lysis curves. However, these lines of evidence could still be regarded as circumstantial, because the tests employed might have created apparent discontinuities even within a single red cell population. For instance, only a fraction of red cells lyse in the autohemolysis test in hereditary spherocytosis, and only a fraction of red cells are irreversibly sickled in sickle cell anemia, even though all the cells clearly belong to one and the same population. In addition, it is not certain that the standard Ham test correctly measures the fraction of PNH cells, because the percentage of cells lysed varies, for instance, with the ionic strength of the medium. Thus, one might still surmise that the PNH abnormality arises as a result of an extracorpuscular factor in a finite fraction of hematopoietic cells. More direct evidence for the monoclonal origin of PNH cells came from the demonstration of only one type of glucose-6-phosphate dehydrogenase (G6PD) in the red cells lysed in acidified serum from two female PNH patients who were heterozygous for two different G6PD variants.

In vitro cultures of erythroid cells now make it possible to test the two-population model in another direct way. This model predicts that erythroid cell-progenitors, such as burst-forming units, erythroid (BFU-E), must also be of two kinds: normal and PNH. Each erythroid burst grown from a single cell would have to consist entirely of either normal or PNH cells. Using two independent criteria, we show that this is indeed the case.

MATERIALS AND METHODS

Patients and Controls

PNH was diagnosed on the basis of chronic hemolytic anemia with hemosiderinuria and a positive Ham test. Patient 1 had never been transfused; and patient 2 had been transfused with RBCs on three occasions, the last transfusion having been given three months before the culture experiments. Peripheral blood mononuclear cells were obtained by venipuncture. In one patient, bone marrow cells were also cultured when an aspirate was performed as part of the patient’s initial hematologic assessment. Mononuclear cells from peripheral blood of some of us served as normal controls.

Erythroid Cell Cultures

Peripheral blood (10 to 30 mL) or bone marrow aspirate (1 mL) was collected with preservative-free heparin (final concentration 10 U/mL). Undiluted blood was layered on an equal volume of Lymphoprep (Nyggaard and Co, Oslo), and centrifuged at 400 g for 40 minutes at room temperature. The mononuclear cell layer was collected and the cells washed twice in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO, Grand Island, NY). The cells were then resuspended in IMDM at a concentration of 5 x 10⁸/mL for peripheral blood and 10⁹/mL for bone marrow. Erythroid cell cultures were set up by the methylcellulose technique, with minor modifications. Briefly, the cell suspension was diluted 1:10 in a mixture containing 0.8% methylcellulose (Methocel 4000 cps, Dow Chemical Co, Midland, Mich), 30% fetal calf serum (Eurobio, Paris), 1% bovine serum albumin (Sigma, St Louis), prepared according to Tepperman et al., beta-mercaptoethanol 10⁻⁴ mol/L and erythropoietin (step III, Connaught, Stillwater, Pa) 2 U/mL.

From the Department of Hematology, Royal Postgraduate Medical School, London; the International Institute of Genetics and Biophysics, Naples; and the Section of Hematology, 2nd Medical School, University of Naples.

Submitted Feb 2, 1984; accepted April 30, 1984.

Address reprint requests to Professor L. Luzzatto, Royal Postgraduate Medical School, Ducane Rd, London W12 OHS, England.

© 1984 by Grune & Stratton, Inc.

0006–4971/84/6404–0014$03.00/O

Blood, Vol 64, No 4 (October), 1984: pp 847–851

847
and was then distributed in 1-mL aliquots in 35 × 10 mm Falcon culture dishes. Cultures were incubated at 37 °C in a humidified atmosphere flushed with 5% CO₂ in air.

Scoring and labeling of cultures was carried out on days 11 to 13. Erythroid colonies were identified on the basis of their orange-red color and their characteristic morphology. In the case of bone marrow, we collected colonies produced from BFU-E (bursts) and not those produced by erythroid colony-forming units (CFU-E).

### Labeling of Cultures

In order to detect lysis of a small number of cells, ³H-leucine was incorporated into erythroid cells at the time of maximal hemoglobin synthesis by a technique similar to that described by Comi et al. Individual erythroid colonies (or bursts), consisting of between 500 and 3,000 cells, were identified on an inverted microscope. A single colony was aspirated through a micropipette and transferred to the bottom of a sterile plastic 10-mL conical test tube containing 10 µL of a mixture of 0.8% methylcellulose in IMDM, 30% fetal calf serum, erythropoietin 2 U/mL, and 10 µL of high specific activity (180 Ci/mmol) ³H-leucine (Amersham). In order to maximize the incorporation of radioactivity, all components of this mixture were dissolved in leucine-free IMDM. The appropriate amount of ³H-leucine had been previously dried under a flow of nitrogen and then dissolved directly in the incubation mixture. Incubation was carried out in a humidified atmosphere at 37 °C for 18 to 24 hours. At the end of this period, 10 mL of phosphate-buffered saline containing 0.5% bovine serum albumin (BSA-PBS) was added to each tube, together with 5 × 10⁶ unlabeled red cells of the same blood group, to act as carrier for the labeled burst. (The use of BSA-PBS as a suspending medium for the washes was in order to avoid unspecified lysis of labeled erythroid cells.) Cells were then washed three times with 10 mL of BSA-PBS, resuspended in 1 mL of the same solution, and transferred to a 1.5-mL Eppendorf conical tube. A 50-µL aliquot was counted. At this stage, bursts with high radioactivity were split into two equal portions, and one of the aliquots was used as control of the specificity of the Ham test (see below). After centrifugation (4,000 rpm, five minutes, in a Beckman Microfuge T11), the supernatant was removed, and 50 µL was counted to ensure that the washing procedure had effectively removed all unincorporated radioactivity.

### Miniaturized Ham Test

The cell pellet from the above step was resuspended in 50 µL of fresh, ABO-compatible acidified (pH 6.5 to 7.0) serum and incubated at 37 °C in a water bath for one hour. After centrifugation (Microfuge, 4,000 rpm for five minutes), the supernatant was collected; the pellet was hemolyzed with 50 µL of distilled water, and centrifuged again (4,000 rpm, five minutes). Radioactivity in both supernatants was determined by counting in 6 mL of scintillation liquid (Ready-Solv MP, Beckman) in a Beckman beta-counter (model 7,000) and was expressed as dpm. Counting efficiency was about 40%. Hemolysis in acidified serum was calculated for each burst as the percentage of radioactivity in the supernatant serum with respect to cytoplasm radioactivity (sum total of ³H released in supernatant serum plus ³H released by lysis in distilled water). The specificity of the miniaturized Ham test was checked by performing tests with heat-inactivated (30 minutes at 56 °C) acidified serum on an aliquot of a burst, the other aliquot being processed with non-heat-inactivated acidified serum.

### AchE Staining

Membrane acetylcholinesterase (AchE) activity was tested on individual colonies by a modification of the method described by Perona et al. A whole burst or part of it (a subcolony) was picked at days 11 to 13 and added to a drop of molten agar (1% in saline) at 40 °C, in a well of a microtiter plate (Micro Test Plate, Nunc, Roskilde, Denmark). After the agar had set, the well was filled with staining mixture. The plate was incubated at 37 °C in humidified atmosphere for 24 to 48 hours. Upon examination under an inverted microscope, positive colonies showed black staining, with the disappearance of cell border and the presence of needle-like crystals, whereas negative colonies showed well-preserved cells without any crystals. Colonies from a normal subject served as positive controls. Omission of acetylthiocholine from the staining mixture served as negative control.

### RESULTS

Both patients had reduced numbers of peripheral blood BFU-Es. A quantity of 5 × 10⁵ mononuclear cells plated yielded 5.35 ± 1.5 and 4.25 ± 1.5 colonies for the patients, compared to 20.1 ± 1.9 in normal controls (P < .001).

Our main objective was to prove that some, but not all, erythroid bursts from PNH patients had the PNH abnormality. Because bursts derived from BFU-E consist of only a few hundreds to several thousand cells, it is not practical to observe lysis of one of them by conventional spectrophotometric analysis of hemoglobin release. However, if individual mature bursts are incubated for 24 hours with a radioactive amino acid (we have used ³H-leucine), this is incorporated into newly synthesized cellular protein—mainly hemoglobin. Cell lysis can then be measured with high sensitivity and accuracy by simply determining the proportion of counts released into the medium. Erythroid colonies at 11 to 13 days consist of mature erythrocytes and of normoblasts, some of which, at least, are susceptible to acid lysis. Erythrocytes grown in vitro are much more fragile than normal ones. However, once suitable conditions are worked out (see Materials and Methods), lysis in acidified serum of individual bursts from normal peripheral blood BFU-Es is mostly below 25%, and it never exceeds 40% (Fig 1). On the other hand, when bursts from peripheral blood or bone marrow BFU-Es of PNH patients are similarly tested, percent lysis ranges from 2.1% to 85.8% (Fig 1). The distribution of bursts according to this property is clearly bimodal (Fig 2). One group of bursts from PNH mononuclear cells is abnormal, and we regard them as PNH bursts. Another group of bursts behaves in a way that is indistinguishable from bursts from normal blood, and we regard them as normal bursts from PNH patients. If an aliquot of an abnormal burst is tested with heat-inactivated serum, percent lysis falls within the normal range, indicating that lysis is complement dependent (Fig 1). Representative data obtained on individual colonies are shown in Table 1. By choosing a cut-off point of 40% lysis, the difference between
NORMAL AND PNH ERYTHROID COLONIES IN PNH

bursts from normal subjects (N = 12) and normal bursts from PNH patients (N = 14) compared to PNH bursts from PNH patients (N = 44) is highly significant ($\chi^2 = 24.68; P < 10^{-7}$).

The activity of AchE, a membrane enzyme, is markedly reduced in most PNH patients.14,15 Perona et al.,11 using cytochemical staining, have demonstrated normal and AchE-deficient erythrocytes in the peripheral blood of PNH patients, suggesting that this enzyme is a good marker of the PNH abnormality. We have found that all erythroid colonies (23/23) from six normal controls were strongly positive by AchE staining. In contrast, by testing single subcolonies from 25 different bursts from a PNH patient, we found 22 of them to be negative (Fig 3A) and only three positive (Fig 3B). The miniaturized Ham test, carried out on the remaining subcolonies from the same 25 bursts, showed that the three positive bursts all had lysis in the normal range, whereas the 22 AchE-negative colonies all had lysis above 45%. No differences were observed between normal and abnormal cultures from PNH patients in terms of size and morphology of colonies and subcolonies, or in the degree of hemoglobinization.

**DISCUSSION**

The finding of two kinds of erythroid cell bursts cultured from PNH patients indicates that there are two kinds of BFU-Es in their bone marrow and peripheral blood. Indeed, if the PNH abnormality were acquired at random by a finite proportion of cells during their differentiation from BFU-Es, then all bursts would include both lysis-susceptible and normal cells in approximately constant proportion, as each is made up of a sufficiently large number of cells to make statistical fluctuation negligible. In this case, bursts from PNH patients would show a unimodal distribution, with an abnormally high modal value of lysis. Instead, a bimodal distribution is observed (Fig 2), and the modal value of lysis of one group of bursts is normal. Thus, normal cells and PNH cells are clearly predetermined at the BFU-E stage.

The evidence presented in this article, combined with the G6PD studies referred to previously, supports Dacie's concept of a clonal population of abnormal erythroid progenitors, which has probably arisen by a single somatic mutation, which generates the PNH erythrocyte population of patients with PNH.

By the experimental procedures we have described, it is possible to obtain a quantitative estimate of the size of the PNH clone in PNH patients. This estimate is probably much more accurate than that based on the percentage of circulating RBCs that are lysed in the Ham test, as with the latter, we may miss the fraction of abnormal cells that has been already lysed in vivo.
and which may be quite substantial. In both patients we have studied, lysis by the Ham test carried out on the patient’s blood at the time of setting up the cultures was about 40%. By testing erythroid colonies, we have found in patient 1 60% abnormal BFU-Es in peripheral blood and 80% in the bone marrow; in patient 2, 88% abnormal BFU-Es were found in peripheral blood. These data are based on the assumption that the plating efficiency for both normal and abnormal BFU-Es from PNH patients is the same. We do not yet know whether this assumption is correct. On the other hand, we are aware that the high proportion of abnormal BFU-Es may be due in part to shortage of normal BFU-E, because a marked decrease in the overall number of erythroid colonies from peripheral blood in PNH patients has been found by ourselves and others.  

Although AchE deficiency is thought to be a secondary phenomenon in PNH, it is characteristically associated with the PNH abnormality because the two populations are seen cytochemically in PNH patients, and the complement-sensitive red cells, physically separated by differential lysis, were found to have practically no AchE activity. Our finding of a close correlation between acidified serum lysis and AchE in individual erythroid colonies confirms that this enzyme is a good marker for PNH. Again, both AchE-positive and AchE-negative colonies are seen, and the match between the two tests employed validates the specificity of both. Because AchE staining is far less laborious than the miniaturized radioactive Ham test, but it appears to be equally sensitive and specific, it could be used as a convenient method to quantitate abnormal BFU-Es in PNH patients.  

After these experiments were completed, Dessypris et al have reported independent evidence that abnormal complement sensitivity can be demonstrated in erythroid and myeloid progenitors in PNH. They have found that erythroid colony numbers were reduced by about 60% when mononuclear cells were incubated with fresh serum and sucrose before plating. Our results agree with those of Dessypris et al in proving that the PNH abnormality is already determined at the BFU-E level. However, these authors have not shown that the BFU-Es killed by fresh serum and sucrose are actually the progenitors of PNH cells, and in this respect, our findings are complementary to theirs.
The role in oncogenesis of somatic mutations makes them most prominent in human pathology, but they are also presumed to play a role in the physiologic process of the generation of antibody diversity. That somatic mutation can be responsible for the production of a veritable acquired mosaicism in a particular cell population is proven by the examples of PNH and of Tn polyagglutinability. PNH has a special relationship to aplastic anaemia and to myeloproliferative disorders, and it might be regarded as a preneoplastic condition. Very recently, a defect in C3 convertase decay-accelerating factor has been discovered in PNH cells, but it is not certain whether this is the primary mutated gene product. The availability of an in vitro culture system for PNH erythroid cells, reported here, may help to test this directly and to explore PNH as a model system of disorders caused by somatic mutation.

ACKNOWLEDGMENT

We thank Dr M. Clarke of St Helier Hospital, Carshalton, Surrey, for providing blood samples from one of the patients, and the patients themselves for their cooperation.

REFERENCES

Two populations of erythroid cell progenitors in paroxysmal nocturnal hemoglobinuria

B Rotoli, R Robledo, N Scarpato and L Luzzatto