Original Article

ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 1 – Clinical Utility

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Paroxysmal nocturnal hemoglobinuria (PNH) arises as a consequence of the non-malignant clonal expansion of one or more hematopoietic stem cells with an acquired somatic mutation of the *PIGA* gene (Brodsky RA. Blood 113 (2009) 6522–6527). Progeny of affected stem cells are deficient in glycosyl phosphatidylinositol–anchored proteins (GPI-APs). This deficiency is readily detected by flow cytometry. Though this seems straightforward, the clinical utility of this testing requires that the ordering clinician understand not only the characteristics of the test, but also the biology of the underlying disease, and the clinical and laboratory manifestations in the individual patient. When interpreted correctly, the results from PNH flow cytometry testing, including presence and size of the clonal populations and the cell types involved, can allow the clinician to classify the disease appropriately; evaluate the risk of disease progression; and subsequently monitor response to therapy. In these guidelines, we discuss the evaluation of a patient with suspected PNH or other bone marrow failure disorders, with specific emphasis on the contribution of this testing to the diagnosis, classification, and monitoring of patients. For convenience we will commonly refer to these flow cytometry studies as "PNH testing" recognizing that an abnormal result is not diagnostic of PNH; rather both laboratory and clinical features are used to establish this diagnosis. © 2017 International Clinical Cytometry Society

Key terms: aplastic anemia; complement inhibitors; paroxysmal nocturnal hemoglobinuria

How to cite this article: Dezern AE and Borowitz MJ. ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 1 – Clinical Utility. Cytometry Part B 2018; 94B: 16–22.

WHO SHOULD BE TESTED FOR PNH?

Initial workup of a patient with suspected PNH includes a complete blood count, reticulocyte count, Coombs assay, and LDH levels. Among these tests, PNH testing is the most sensitive and specific test available. However, it should not be used as a screening test patients presenting with anemia. Common causes of anemia should be excluded *prior* to flow cytometric testing for PNH.

It is clinically appropriate to send a PNH test in patients with laboratory markers of hemolysis such as elevated lactate dehydrogenase (LDH) or a reticulocytosis when other more common causes of hemolysis have been excluded. Additionally, young patients with unexplained thromboses or patients diagnosed with thromboses in an unusual site (e.g., intra-abdominal veins and arteries, cerebral veins, dermal veins) should also be considered for PNH testing, especially when there is concurrent evidence of hemolysis, no matter how minor. PNH testing should not be part of the first-

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Received 3 October 2017; Revised 6 December 2017; Accepted 7 December 2017

Published online 13 December 2017 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/cyto.b.21608

line workup of a patient with an uncomplicated or triggered deep-vein thrombosis. Finally, it is essential to recognize that PNH testing may be informative in several non-PNH conditions, such as inherited or acquired aplastic anemia (AA), and myelodysplastic syndrome (MDS). Accordingly, patients with unexplained cytopenia in whom AA or MDS are differential diagnostic considerations should be tested for PNH at the time of diagnosis.(1)

As detailed elsewhere, flow cytometric detection of PNH clones requires staining cells with monoclonal antibodies directed at various GPI-APs. A particularly useful reagent is a fluorescein-tagged proaerolysin variant (FLAER) that binds the glycan portion of the GPI anchor (2). Distinct phenotypes of PNH erythrocytes may be identified according to their sensitivity to complement-mediated lysis in vitro (3,4). Erythrocytes with only modest hypersensitivity (3–5 times normal values) or a more pronounced hypersensitivity to complement-mediated lysis (15–25 times the normal one) can typically both be detected in PNH patients and are known as PNH type II and type III erythrocytes (4). By flow cytometry, these correspond to cells with a partial or complete deficiency of GPI-APs, respectively (5). Because of the complement sensitivity of red cells to lysis, PNH red cell clones do not reflect the full extent of the disease in many patients; measurement of neutrophil and monocyte clone percentages better reflect the size of the PNH clones. (Note that we use the more appropriate term "neutrophils" instead of the historical term "granulocytes" throughout this document. Modern gating techniques based on light scatter, CD45 and CD15 (see parts 2 and 3) result in exclusion of basophils and most eosinophils (although not bands) from the population of interest so that for the most part "granulocyte" clones are in fact "neutrophil" clones. Nevertheless, some figures may still be labeled as granulocytes or "grans.")

INTERPRETATION OF PNH TESTING RESULTS

The Significance of the Absence of a PNH Clone

To interpret a report correctly, a clinician must be aware of the sensitivity of the assay in that laboratory. If the report states that no PNH clones can be detected in either red blood cells or white blood cells he or she can feel assured that PNH has been ruled out even if the sensitivity of the assay is no better than about 1%. However, such a test provides no information to inform a possible diagnosis of AA. A negative result is only useful to the extent that it is obtained with a modern high sensitivity flow cytometry assay that can achieve lower limits of quantitation of 0.01%. While, while not ruling out AA, such a result at least might make a clinician investigate further for other potential causes of primary bone marrow failure, such as inherited disease. As discussed below, sensitivities much beyond this may not be clinically relevant.

The Significance of the Presence of a PNH Clone

Some studies have assumed that the presence of GPI-AP deficient blood cells at any level is a reliable surrogate for the presence of a PNH clone, but caution should be applied in this interpretation. Although larger clones always reflect a marrow stem cell abnormality, some have considered populations of GPI-AP -deficient neutrophils as low as 0.003% to be abnormal (6). However, a threshold this low may lack specificity. Simultaneous measurement of the presence of GPI-AP deficient cells by peripheral blood flow cytometry and a functional bone marrow BFU-E assay under proaerolysin selection allowed for PIG-A gene sequencing and proof of clonality at a stem cell level. These studies allowed both detection and characterization of very small PNH clones in health and disease (7). In AA and PNH, GPI-AP deficient cells in arise from a multipotent HSC; PIG-A mutations in these patients are clonal and affect all hematopoietic lineages, including T cells. In contrast, GPI-AP deficient cells that can be detected either in healthy controls or patients with MDS arise from colony forming cells (CFC); the PIG-A mutations in these individuals are transient, non-clonal, and do not involve T cells (7). These studies demonstrate fundamental differences between AA and MDS and help explain why PNH may arise from AA but not from MDS. Furthermore, in healthy controls the PIG-A mutation rate in CFC assays was demonstrated to be about 1 in 50,000 ($\sim$ 0.002%) (7) while that of PNH RBC phenotypes is 2–6 per million (i.e., 0.002–0.006%) in fresh PB of normal individuals. Thus, for diagnostic purposes, a threshold of 0.01%, which represents a level about 5–10X above that ever detected in healthy controls appears to be clinically reasonable threshold to define a disease-related PNH clone (2).

Interpretation of a positive result requires that a clinician pay further attention to the granularity of the report, as it is important to note the presence of either or both a PNH red blood cell clone and a PNH white blood cell clone. Also of importance are the percentages of Type III cells and Type II red cells as the well as the percentage of the PNH white blood cell clones in neutrophils and monocytes. Consideration of results, in the context of the clinical picture, can help the clinician to diagnose and classify disease, assess risk of progression and thrombosis, and monitor patients while on therapy.

CLASSIFICATION OF PATIENTS WITH ABNORMAL PNH TESTING RESULTS

The International PNH Interest Group (IPIG) has classified disease with PNH clones into 3 subtypes: *classical PNH*, which includes hemolytic and thrombotic patients who have evidence of PNH clones in the absence of another bone marrow failure disorder; *PNH in the context of other primary bone marrow disorders*, such as AA and MDS; and *subclinical PNH*, in which patients have small PNH clones but no clinical or laboratory evidence of hemolysis or thrombosis (8). Distinction among these may be challenging in some clinical scenarios (see below) especially because there are varying degrees of marrow failure in all settings of PNH; it has been demonstrated that PNH patients have a reduced number of hematopoietic progenitors assessed by CFC assays regardless of how they are classified (9,10).

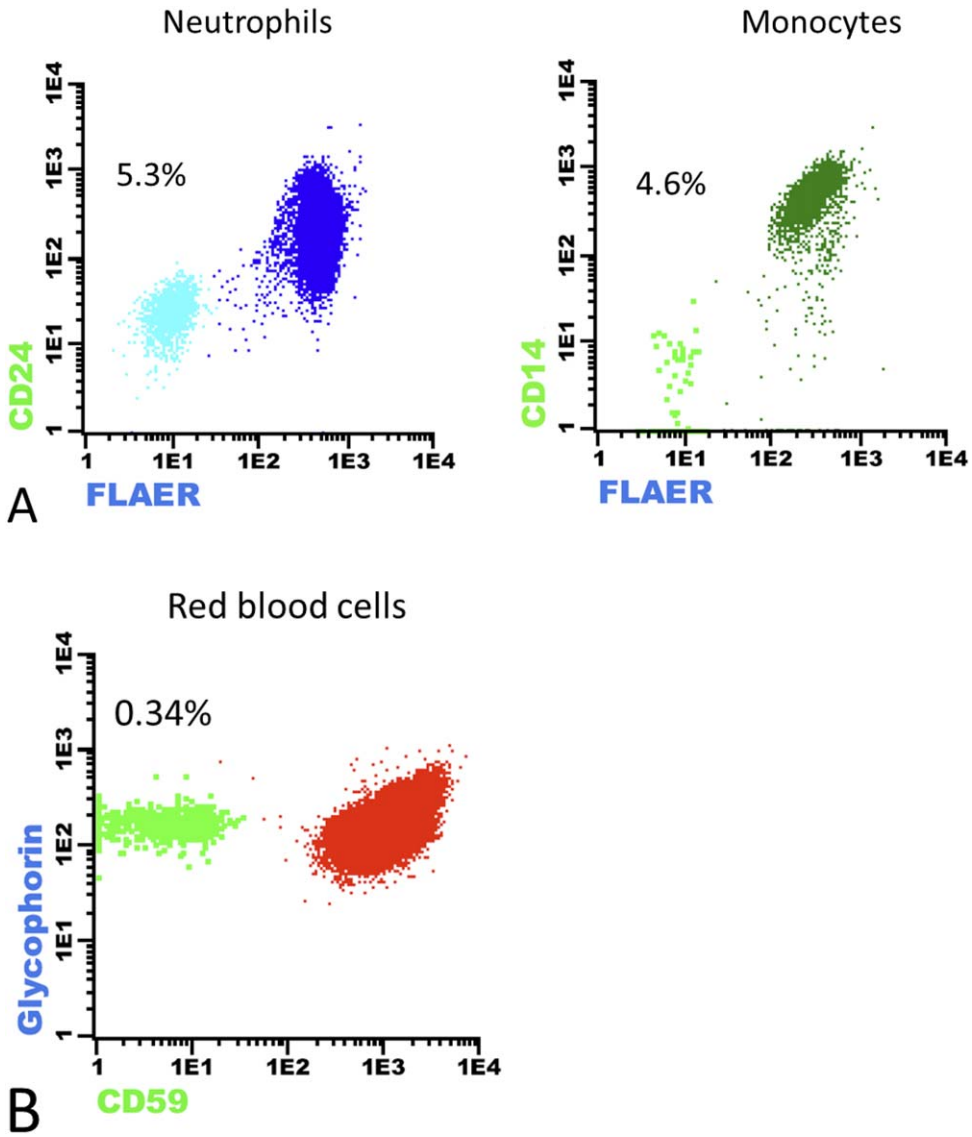

FIG. 1. **A.** Lysed peripheral blood from patient described in Case 1 at diagnosis, gated using light scatter and bright CD15/dim CD33 to identify neutrophils (left) and bright CD33/dim CD15 to identify monocytes (right). PNH neutrophil population identified as CD24 and FLAER negative; PNH monocyte population identified as CD!4 and FLAER negative. **B.** Unlysed peripheral blood from same patient gated on scatter and glycophorin A. PNH (type III) red cells identified as CD59-negative glyophorin positive cells.

Nevertheless, the IPIG classification is an appropriate guideline to use at the time of initial diagnostic testing for contemplating a patient's initial therapy, and assessment of clone sizes in both red and white cell populations can help in this characterization. However, because distinction among categories may be challenging in a particular given clinical situation, this classification alone cannot directly dictate management.

Patients with classical PNH typically have higher clone sizes in WBCs at the time of diagnosis than patients in other categories; these patients also by definition have evidence of hemolysis in the form of elevated LDH and reticulocyte counts. Although monocyte and neutrophil/granulocyte clone sizes typically correlate well, discrepancies may be noted, rarely to an extreme degree. Except in extreme cases, these discrepancies rarely change clinical practice. Red blood cell clone sizes, however, are less reliable as these may be small relative to the white cell clones either because of brisk hemolysis or because of transfusions shortly before the assay was performed. Importantly, the size of the neutrophil/granulocyte clone is relevant to the thrombosis risk for the classical PNH patient (11). Patients with subclinical PNH have the smallest clone sizes of any of the IPIG classification groups

The PNH registry contains substantial information about clone sizes in different groups of patients, including AA patients, although it is not possible to correlate the data precisely with the IPIG classification. The median granulocyte clone size in the first 1,600 patients

enrolled in the PNH registry was 68.1% (range 0.01–100%). This was larger in patients with classical PNH than in patients with AA (83% vs. 35%; $P > 0.001$) (12). Clone sizes in PNH in the context of other primary bone marrow failure (BMF) disorders, such as AA and MDS are highly variable. In the PNH Registry data, distribution of clone sizes in such patients varied considerably: 34% of patients had clone sizes of 50% or higher, while 40% of patients had clone sizes <10%. By contrast, 76% of patients with PNH but without BMF had clone sizes > 50% but only 8% had clone sizes < 10%. Although these differences are clear when one looks at population data, clinically there is considerable overlap between PNH and AA, as they have long been viewed as distinct forms of the same disorder (13).

The leading hypothesis to explain the relationship between AA and PNH is that the autoimmune attack of AA preferentially targets normal HSC over PNH HSC (14). Up to 70% of patients with acquired, but not inherited, AA have small, clonal, populations of PNH cells at diagnosis (15), and up to 40% of PNH evolves from AA. Whether GPI-AP-deficient cells predict therapeutic response in AA is debated (6,16). PNH testing may be particularly useful in young patients presenting clinically with aplastic bone marrows, as PNH clones are not seen in inherited causes of aplastic anemia; thus identifying a clone may prevent the need for more extensive genetic testing (17). Patients with AA may have clinically silent small PNH populations for many years but expansion of the PNH clone often occurs, particularly at the time of relapse of the disease. It is particularly important to follow a PNH clone serially in AA patients (18). Patients with no detectable clone should be screened every 6 months, decreasing to yearly if no clone appears in the first two years. If a clone is present or appears, patients should be screened every 3 months until the clone size is shown to be stable for 2 years. There is no role for serially monitoring the size of the PNH clone in MDS patients. When serial testing is performed, it can be useful for the report to note the relative difference from previous testing.

ILLUSTRATIVE CASES DEMONSTRATING UTILITY OF PNH CLONE TESTING

Using PNH Clone Size in Older Patients to Aid in the Differential Diagnosis of Acquired MDS

Patient 1: 68-year old female with pancytopenia treated with azacitidine for myelodysplastic syndrome. She was referred for treatment-refractory MDS due to protracted time to count recovery. PNH testing revealed a red blood cell clone of about 0.3% and a PNH white cell clone of 5.3% on neutrophils and 4.6% on monocytes (See Figure 1). Her marrow had always been hypocellular for age. Her karyotype was 47XX, +8.

DISCUSSION: In an older patient, MDS is much more common than aplastic anemia, but a clone size in this range is unusually large for MDS. Because of our suspicion of AA, based on the larger clone size, her therapy was switched from azacitidine to immunosuppressive therapy (IST) and her peripheral blood counts increased to near normal. Presence of a nonspecific cytogenetic abnormality such as trisomy 8 cannot, by itself, exclude an immunologic mechanism contributing to cytopenia.

Patient 2: 67-year old male with cytopenias and concern for suppression from antibiotics from a skin infection. He refused bone marrow evaluation until further evidence was provided to justify its clinical relevance. PNH testing revealed a red blood cell clone of 0.02% (not shown), and a white cell clone of 0.21% on neutrophils and 0.17% on monocytes. (Figure 2).

DISCUSSION: Because we consider finding a small abnormal PNH clone to be evidence of a primary marrow disorder, we were able to convince the patient of

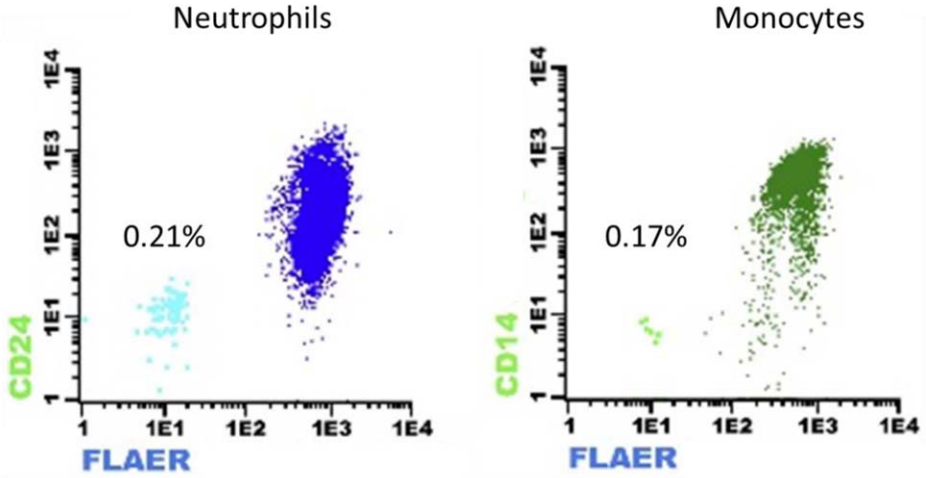

FIG. 2. Lysed peripheral blood from Patient 2 gated and analyzed as described in Figure 1A above both small neutrophil and small monocyte PNH clones. [Color figure can be viewed at wileyonlinelibrary.com]

the importance of a marrow examination. Results of this marrow revealed monosomy 7 and established a diagnosis of MDS.

Marker of Clonal Evolution versus Relapse on Serial Testing in Young Acquired AA Patients

Patients 3 and 4 were both previously healthy children who presented with pancytopenia and hypocellular bone marrows.

Patient 3: 16-year old male who presented with pancytopenia and had a PNH red blood cell clone of 0.58% (0.48% Type III cells and 0.10% Type II cells) at diagnosis, one day following a red cell transfusion. A PNH white blood cell clone was detected in 15.6% of neutrophils and 10.5% of monocytes. There was no evidence of hemolysis. The patient lacked an available matched sibling donor and was treated with IST to which he had a complete hematologic response with a normocellular marrow at 6 months. At that time a PNH clone comprised 1.4% of neutrophils and 1.2% of monocytes. This was monitored serially q3–6 months without significant change and IST tapered. His pancytopenia (most significantly anemia) redeveloped at 18 months post IST. It was presumed that his SAA was relapsing and he was placed back on IST without count augmentation. At a 2-year evaluation, a PNH red blood cell clone was 22% (20% Type III cells and nearly 2% Type II cells) and a PNH white blood cell clone was detected in 82% of neutrophils and 79% of monocytes (Figure 3).

DISCUSSION: In spite of the relatively high WBC clone at diagnosis the patient did not have evidence of classical PNH and responded to IST. The subsequent very high PNH white blood cell clone, and his 22% red blood cell clone were higher than would have been expected in simple relapse of SAA and rather suggested conversion to classical PNH. In support of this diagnosis, an LDH was found to be 1200 and a bone marrow was now hypercellular. Eculizumab was started and his anemia resolved. Repeat PNH testing showed his red blood cell and white blood cell clone sizes were unchanged after 6 months on therapy, but by 9 months of therapy he was in complete remission, with no detectable red or white blood cell clones as shown in Table 1.

Patient 4: 15-year old female who had a detectable PNH clone at diagnosis (7 days post red cell transfusion): red blood blood cell clone 0.05% (all Type III cells) and a white blood cell clone of 0.87% of neutrophils and 0.9% of monocytes. This patient also lacked a donor and had complete hematologic response to IST with a normocellular marrow at 12 months. PNH clone testing at that time detected a white blood cell clone in 0.6% of neutrophils and 0.013% of monocytes. This was monitored serially q6mos without significant change in her blood counts. At 18 months post IST, the clone was noted to have increased to greater than pre-IST value: 0.87% of neutrophils and 1.94% of monocytes. Though her counts remained at the same levels post IST, she was told her SAA could be relapsing and the frequency of her monitoring was increased. Within 4–6 weeks, she again became transfusion dependent and PNH testing showed a PNH red blood cell clone of 1.5% (1.3% Type III cells). A PNH white blood cell clone was detected in 4% of neutrophils/granulocytes and 8% of monocytes. An LDH was then checked and found to be 220 even in the face of an increase in her white cell clone size. A marrow was again hypocellular. All these findings are consistent with relapse of her SAA rather than progression to PNH

DISCUSSION: The frequency of monitoring was altered here as the increasing PNH close size heralded relapse. This proactive approach allowed for early detection and accurate diagnosis of relapse. Both the clinician and the patient were prepared to avoid any dangerous sequelae at the time of relapse. Continued monitoring is important as the risk to conversion to PNH remains.

Documentation of the presence of PNH clones in both patients at diagnosis supported the diagnosis of acquired AA and prevented extensive genetic work up for inherited causes of the anemia.

Marker of Response to Eculizumab Therapy

Three patients receiving eculizumab therapy for classical PNH showed varied responses to the therapy as shown in Table 1.

Table 1
Different patterns of response to eculizumab in three patients

Age/sex	Clinical response	Length of Dx (in months)	Pre-tx Hb (g/dL)	Post-tx Hb (g/dL)	Pre-tx LDH (U/L)	Post-tx LDH (U/L)	Pre-tx Retic (K/cu mm)	Post-tx Retic (K/cu mm)	Pre-tx RBC clone size (%)	Post-tx RBC clone size (%)	Pre-tx Gran clone size (%)	Post-tx Gran clone size (%)
46 M	Persistent anemia	3	8.1	9.1	1,551	200	139.7	222.9	32	50	83	94
27 M	Improve-ment in hemoglobin	2	8.9	12.7	6,218	329	180	164.6	6.66	49	73	87
33 M	Normal-ization of hemoglobin	13	8.6	17.4	1,670	274	NA	133.5	46	6.9	87	24

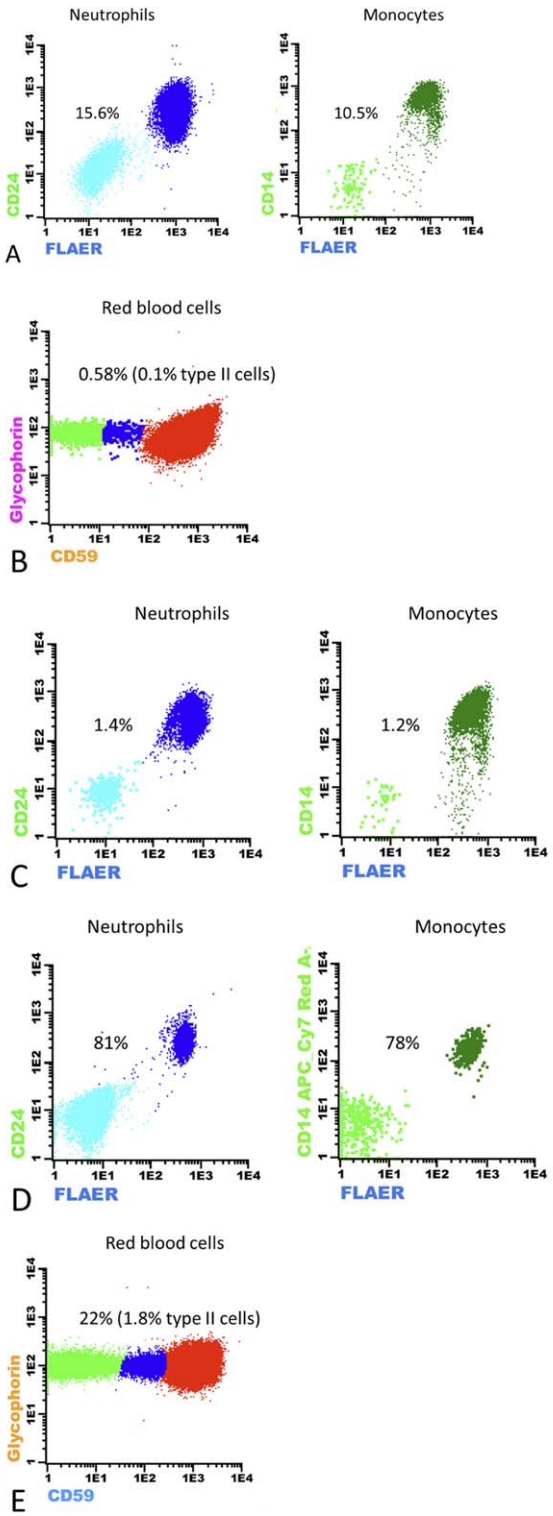

FIG. 3. Specimens from Patient 3 processed and analyzed as described in Figure 1. **A and B**. White cell and RBC clones detected at the time of presentation with pancytopenia. **C**. Same patient following immunosuppressive therapy, after hematologic remission, showing a significant decrease in the neutrophil and monocyte clones. **D and E**. Same Patient 18 months later following recurrence of pancytopenia, showing development of frank PNH with large white cell clones and significant RBC clone.

DISCUSSION

Following a PNH clone serially in classical PNH is useful because clone size may correlate with disease responsiveness. If the patient is stable, PNH clones may be monitored yearly in this group. Normalization of the hemoglobin levels appears to predict for a decrease in the size of the red cell clone (19). The three patients demonstrate examples of the types of responses seen in patients treated with eculizumab. Many classical PNH patients can become red cell transfusion independent. Typically, following successful therapy the RBC clone shows a paradoxical increase in size because eculizumab blocks hemolysis of the GPI-AP-deficient PNH red cells. However, most responders continue to have laboratory evidence of extravascular hemolysis, with mild to moderate anemia and a persistently elevated reticulocyte count. This in turn may lead to a decrease in the size of the red cell clone. This occurs because the extravascular hemolysis in PNH is mediated via C3 deposition on PNH red cells, but not normal red cells, so that the former are selectively destroyed. In fact, this drop in RBC clone size may be linked to more robust responses, as with the response in the table above. Thus, the pattern of PNH red blood cell clone changes may predict for improvement or normalization of a patient's blood counts, or even spontaneous remission of the disease, although interpretation of results also depends on the transfusion status and the level of ongoing hemolysis (19). For these reasons, the entire clinical picture of a patient's course must be taken into account when interpreting PNH clone size measurements.

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