

Bone Marrow as a Source of Cells for Paroxysmal Nocturnal Hemoglobinuria Detection

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ABSTRACT

Objectives: To determine fluorescently labeled aerolysin (FLAER) binding and glycosphosphatidylinositol–anchored protein expression in bone marrow (BM) cells of healthy volunteers and patients with paroxysmal nocturnal hemoglobinuria (PNH) detected in peripheral blood (PB); compare PNH clone size in BM and PB; and detect PNH in BM by commonly used antibodies.

Methods: Flow cytometry analysis of FLAER binding to leukocytes and expression of CD55/CD59 in erythrocytes. Analysis of CD16 in neutrophils and CD14 in monocytes in BM.

Results: FLAER binds to all normal BM leukocytes, and binding increases with cell maturation. In PNH, lymphocytic clones are consistently smaller than clones of other BM cells. PNH clones are detectable in mature BM leukocytes with high specificity and sensitivity using common antibodies.

Conclusions: PNH clone sizes measured in mature BM leukocytes and in PB are comparable, making BM suitable for PNH assessment. We further demonstrate that commonly used reagents (not FLAER or CD55/CD59) can reliably identify abnormalities of BM neutrophils and monocytes consistent with PNH cells.

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematologic disorder caused by somatic mutations in the phosphatidylinositol glycan class A (*PIG-A*) gene.¹ The product of this gene takes part in the biosynthesis of the glycosphosphatidylinositol (GPI) anchor,²⁻⁵ a molecule that couples numerous proteins to the cell membrane. Because *PIG-A* gene mutations originate in a multipotent hematopoietic stem cell (HSC) with differentiation and self-renewal capacity, all progenies derived from the mutated HSC harbor the GPI defect and have a complete or partial deficiency in the expression of GPI–anchored protein (AP)^{6,7} on the cell surface. Some of these GPI-APs, such as CD55 and CD59, are regulators of the complement cascade by interfering with the formation and stability of the C3 convertase (CD55) or with the assembly of the terminal complement complex (CD59).⁸ In this manner, red blood cells (RBCs) of healthy individuals are protected from complement-mediated destruction, whereas those deficient in CD55 and CD59 are sensitive to intra- and extravascular hemolysis.⁹ PNH is often associated with acquired bone marrow failure syndromes, such as idiopathic aplastic anemia (AA) and myelodysplastic syndromes (MDS). In AA, up to 60% of patients harbor small to moderate PNH populations,^{10,11} whereas in MDS, the prevalence of PNH clones is lower, 10% to 15%.^{12,13}

Flow cytometric (FC) analysis of GPI or GPI-AP expression on leukocytes (neutrophils) or RBCs from peripheral blood (PB) is currently the method of choice for the laboratory diagnosis of PNH. Traditionally, most

FC analyses have focused on testing the expression of the two GPI-APs, CD55 and CD59. A simple method using a fluorescently labeled inactive variant of the protein aerolysin (FLAER) that directly binds to GPI anchors emerged as a superior method and became a new standard for PNH testing in granulocytes and monocytes.¹⁴

PB RBCs and white blood cells (WBCs) have been extensively studied in PNH, but there have been only few efforts to delineate in detail the abnormalities of bone marrow (BM) cells in patients with this disorder.^{15,16} BM specimens are generally considered less suitable than PB owing to variable expression of GPI-AP during the various stages of hematopoietic cell development and are seldom evaluated for PNH. However, BM aspirates from patients with unexplained cytopenias, including BM failure syndromes, are frequently submitted to laboratories for general diagnostic purposes, but targeted PNH analysis is infrequently performed on these samples.¹⁶

In our laboratory, we receive a large number of BM samples from patients referred for cytopenias. Most of these patients are diagnosed with AA, and a minority has MDS. As expected, a significant proportion of patients with AA and MDS carry PNH clones of different sizes discovered by blood FC-based PNH assays. Our patients are followed long term and are frequently tested for PNH. This provided us with a unique opportunity to investigate BM PNH cells in patients with acquired BM failure and compare our results with measurements on cells from PB.

FLAER has not been thoroughly investigated in BM.¹⁵ Using the power of FC, we analyzed patterns of FLAER binding to myeloid and lymphoid cells and CD55/CD59 expression on nucleated RBCs (NRBCs) in BM aspirates of healthy volunteers and patients with detectable PNH cells in the PB. In these patients, we also compared the clone size measured by FLAER and CD55/CD59 antibodies in BM leukocytes and NRBCs, with the PNH clone size determined in circulating neutrophils. In addition, we demonstrated that BM analysis performed by FC with routinely used antibodies such as CD45, CD64, CD13, CD11b, and the GPI-APs CD14 and CD16 (but not FLAER or CD55 or CD59) identifies phenotypic abnormalities in granulocytes and monocytes that are consistent with the presence of PNH clones with high sensitivity and specificity.

Materials and Methods

BM Samples

Samples were selected from patients enrolled in institutional review board–approved National Heart, Lung, and Blood Institute protocols for treatment of acquired

BM failure, mostly AA, and from healthy volunteers. All patients or their legal guardians provided informed consent. Idiopathic AA and its severity are defined by various degrees of multilineage cytopenia, low BM cellularity of less than 30%, and absence of dysplasia. A minority of patients were diagnosed with MDS, following the World Health Organization 2008 criteria, one with single-lineage dysplasia (refractory anemia) and another with multilineage dysplasia (refractory cytopenia with multilineage dysplasia). As part of a diagnostic algorithm, these patients were screened for the presence of PNH clones in the PB and underwent serial BM aspirate and biopsy examinations to establish diagnosis and monitor disease and response to treatment. A portion of BM aspirate sample from patients with PNH clones in PB neutrophils equal or higher than 1% were used for our BM studies involving FLAER and CD55/CD59 staining. As controls, we studied 11 BMs from healthy volunteers. All samples were received fresh, anticoagulated with EDTA, and processed within 24 hours.

To determine whether routine immunophenotypic analysis of BM by FC using common antibody panels could identify PNH clones without the use of PNH-specific markers, we analyzed neutrophils and monocytes in BM aspirates from 50 patients with and 105 without PNH clones detected in PB by standard PNH markers. In this study, a detectable clone was defined as the presence of 1% or more PNH cells. Monocytes in 15 samples could not be analyzed due to low monocyte counts.

Flow Cytometry

All samples were initially stored at 2°C to 8°C but processed at room temperature. Following two washes in phosphate-buffered saline-albumin (PBA), the BM aspirate cell counts were adjusted to contain 5×10^5 to 10^6 cells in 100 μ L PBA per tube. Appropriately titrated antibodies and FLAER (see list in **Table 1**) were then added to each tube in a combination that allowed the analysis of FLAER binding on individual cell lineages and stages of development. After incubation in the dark for 15 to 30 minutes at room temperature, cells were treated with FACSLyse (BD Biosciences [BD], San Jose, CA) to lyse RBCs, followed by fixation (BD Stabilizing Fixative; BD).

Flow Cytometry Analysis

Data were acquired on a FACS Canto II flow cytometer (BD) equipped with three lasers and 10 (eight light colors and two scatter) detectors using DIVA software (BD). Data analysis was carried out by FCS Express (De Novo Software, Glendale, CA). A minimum of 100,000 events were collected for each tube. Daily instrument performance check was performed using the CS&T module

Table 1
List of Conjugated Antibodies and Fluorescently Labeled Aerolysin Reagents Used for Flow Cytometry

Antibody	Conjugate	Source ^a	Catalogue No.
CD3	PE-Cy7	BD Pharmingen	557749
CD10	APC	BD Biosciences	340922
CD11b	APC	BD Biosciences	340937
CD13	PE	BD Biosciences	340686
CD14	APC-H7	BD Biosciences	641394
CD16	FITC	BD Biosciences	555406
CD19	PE-Cy7	BD Biosciences	341103
CD20	Pacific Blue	Beckman Coulter	A74777
CD33	PerCP-Cy5.5	BD Biosciences	341650
CD34	PE-Cy7	BD Biosciences	348801
CD34	APC-Alexa 750	Beckman Coulter	A89309
CD38	APC-Alexa 750	Beckman Coulter	A86049
CD45	V500	BD Biosciences	560777
CD64	PE	Invitrogen	CD6404-4
CD71	APC-Alexa 700	Beckman Coulter	A97051
CD117	PerCP-Cy5.5	BD Biosciences	333944
CD123	APC	BD Biosciences	560087
HLA-DR	Pacific Blue	Biolegend	307633
CD55	PE	BD Biosciences	555694
CD59	PE	BD Biosciences	555764
FLAER	Alexa 488	Cedarlane Laboratories	FL2S-C

^aBD Pharmingen, Franklin Lakes, NJ; BD Biosciences, San Jose, CA; Beckman Coulter, Indianapolis, IN; Invitrogen, Carlsbad, CA; Biolegend, San Diego, CA; Cedarlane Laboratories, Burlington, Canada.

and beads (BD). Spectral compensation was performed with Compbeads (BD).

Following exclusion of cell doublets/aggregates, debris/platelets, and small cell fragments using forward light-scatter (FSC) parameters, the binding of FLAER was analyzed on the following cell lineages and developmental stages (identified by the indicated parameters):

- All granulocytes: Side light scatter (SSC)/CD45
- Myeloblasts: SSC/CD34/CD13/CD117
- Neutrophils (promyelocytes, myelocytes, metamyelocytes/bands, and mature neutrophils), and eosinophils: SSC/CD45/CD13/CD11b/CD16/HLA-DR
- Monocytes (immature and mature monocytes): SSC/CD64/CD33/CD14
- Plasmacytoid dendritic cells: SSC/CD45/CD123/HLA-DR
- Basophils: SSC/CD45/CD123/HLA-DR
- B cells (lymphoblasts, intermediate precursors, and mature B cells): SSC/CD45/CD34/CD19/CD20/CD10
- Plasma cells: FSC/CD45/CD38
- T cells: SSC/CD45/CD3/CD19
- NK cells: SSC/CD45/CD56/CD3/CD19

Since RBCs do not bind FLAER adequately, we analyzed the expression of CD55/CD59 on NRBCs, which were identified by SSC/CD45/CD71.

The FLAER binding to normal marrow cells was expressed as the ratio between the mean fluorescence

intensity of the population of interest and that of T lymphocytes from the same sample, which were used as an internal control.

PNH clone size was measured as the fraction of cells with decreased or absent FLAER binding or CD55/CD59 expression (GPI defective) for WBCs and RBCs, respectively, compared with that of normal cells. In cases with more than one GPI-negative population present (phenotypically described as type II and type III),¹⁷ no attempt was made to separately quantify the subclones, which were measured in aggregate. The PNH clone size in PB leukocytes was determined separately and ranged from 1% to 99%.

For the detection of PNH cells in BM analyzed with antibodies used for routine diagnostic purposes, we separately analyzed CD16 expression on mature neutrophils and CD14 expression on monocytes. Mature neutrophils were identified based on SSC, CD45, CD13, and CD11b. A minimum of 2,000 mature neutrophils were required to detect a cluster of 1% or more of CD16-negative mature neutrophils. Monocytes were identified based on SSC and their intense CD64 (and/or CD33) expression, and only cases with sufficient numbers of CD14-negative monocytes (≥ 200 events) were analyzed in this manner.

Results

Binding of FLAER to Normal BM Cells

Using appropriate antibody combinations in conjunction with SSC signals, it was possible to identify the major bone marrow cell lineages and also, in some lineages, determine different stages of cell differentiation.

Neutrophils

During the differentiation of neutrophils, FLAER bound to all stages of maturing precursors (defined according to CD13 and CD11b expression)¹⁸ **Figure 1A**. As these cells differentiated, there were changes in the binding of FLAER: promyelocytes and myelocytes showed generally dimmer and bimodal binding of FLAER, whereas metamyelocytes/bands and mature granulocytes bound FLAER more homogeneously (unimodally) and more intensely **Figure 1B**. FLAER binding to myeloblasts was lower than differentiating granulocytes and heterogeneous (bimodal or multimodal distribution) (**Figure 1B**).

Monocytes

Normal monocytes also showed a progressive increase of FLAER binding as they underwent maturation, based on CD14 expression **Figure 2**. Both mature and immature monocytes showed homogeneous FLAER binding distribution.

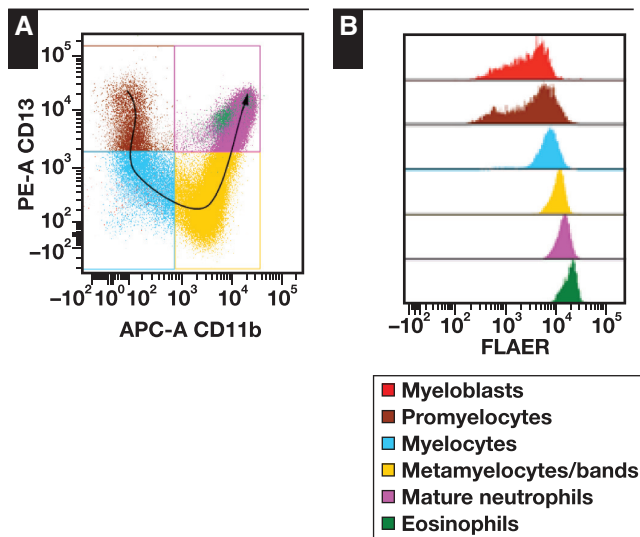


Figure 1 Fluorescently labeled aerolysin (FLAER) binding to normal granulocytes. **A**, Dot plot showing the CD11b and CD13 expression on granulocytes that were selected based on their CD45 expression and side light-scatter properties (not shown) in a bone marrow sample from a healthy adult. This combination of antibodies allows the delineation of neutrophil maturation stages ranging from the least mature to the most mature immunophenotype (arrow): promyelocytes (brown), myelocytes (light blue), metamyelocytes/bands (yellow), and mature neutrophils (magenta).¹⁸ Eosinophils are shown in green. **B**, FLAER binding to different maturation stages of neutrophils and to eosinophils. There is increased fluorescence from FLAER binding as neutrophils mature. Myeloblasts (top histogram) were selected separately based on their coexpression of CD34, CD13, and CD117, but natural killer cells showed less staining overall.

Lymphocytes

There was expression of FLAER receptors during all differentiation stages of B lymphocytes. This expression gradually increased from lymphoblasts (CD19+/CD34+/CD10+/CD20-) along all intermediate stages of maturation, as noted in **Figure 3A**, reaching the highest level in mature B lymphocytes **Figure 3B**. In contrast to myeloblasts and early neutrophils, early lymphocytes, including B lymphoblasts, bound FLAER more homogeneously (**Figure 3B**). Among normal mature lymphoid cells, all T cells and B lymphocytes showed FLAER staining of similar intensities, but NK cells showed less staining overall.

Other Granulocytes and Dendritic Cells

All eosinophils showed slightly brighter fluorescence than other granulocytes, whereas basophils showed the least FLAER fluorescence among all granulocytes **Figure 4**. Plasmacytoid dendritic cells demonstrated a lower FLAER binding than that of mature monocytes (**Figure 4**). Other dendritic cell types were not studied.

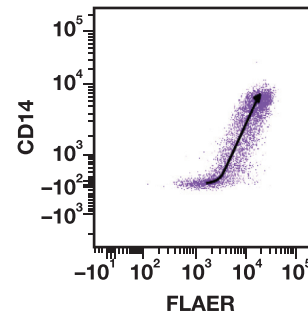


Figure 2 Fluorescently labeled aerolysin (FLAER) binding to normal monocytes. Dot plot showing monocytes in a bone marrow sample from a healthy adult. The monocytes were selected based on their intense CD64 expression and side light-scatter properties (not shown). As monocytes mature, their CD14 expression increases (arrow), and there is increased fluorescence from FLAER binding.

Plasma Cells

Compared with lymphocytes, the binding of FLAER to plasma cells was relatively poor (**Figure 4**).

Summary Chart

The bar chart shown in **Figure 4** indicates the relative fluorescence from FLAER staining of the different populations studied.

Binding of FLAER in BM With PNH Clones

In patients with detectable PNH cells in the blood, we showed that, in comparison to their normal counterparts, most BM populations demonstrated loss of GPI-APs that was identified by weak or absent FLAER. These GPI-negative cells comprised what is designated as the PNH clone. The presence of GPI-negative cells was demonstrated in all maturation stages of neutrophils **Figure 5**, eosinophils (**Figure 5**), basophils, monocytes, dendritic cells, and all stages of B-lymphocyte development **Figure 6**. Myeloid cells showed higher proportions of GPI-defective cells than mature lymphocytes. Among mature lymphocytes, in most cases, the clone size was largest in the natural killer (NK) cells, followed, in descending order, by B cells and T cells. Plasma cells showed very small or absent populations of GPI-negative cells.

In most BM subpopulations, the GPI-negative cells could be distinguished from the GPI-positive cells. However, in early granulocytes, including myeloblasts, the PNH cells (GPI negative) could not be clearly distinguished from the immature cells that normally show poor FLAER binding. GPI-negative NK cells were clearly separated from normal counterparts. Similar to what has been previously described for blood cells,^{19,20} some cases

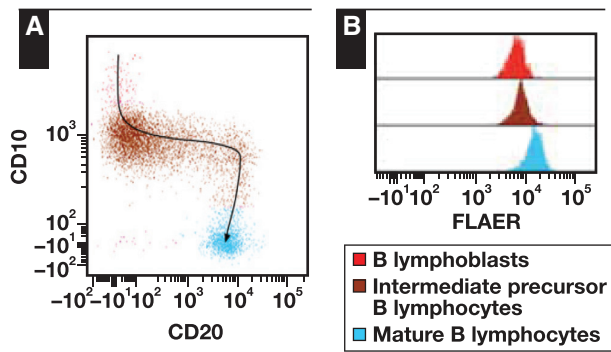


Figure 3 Fluorescently labeled aerolysin (FLAER) binding to normal B lymphocytes. **A**, Dot plot showing CD20 and CD10 expression on lymphocytes in a bone marrow sample from a healthy adult. Only B lymphocytes are shown, which are selected based on their CD19 expression and side light-scatter properties (not shown). This combination of antibodies allows the delineation of three different B-lymphocyte maturation stages ranging from the least mature to the most mature immunophenotype (arrow): early B-cell precursors (red), intermediate B-cell precursors (brown), and mature B cells (light blue). **B**, Histograms depict FLAER fluorescence from the corresponding cell subpopulations shown on dot plots in Figure 3A. Lymphoblasts (top histogram) were selected separately based on their coexpression of CD19 and CD34.

revealed more than one GPI-deficient subpopulations best observed among granulocytes (Figure 5).

Correlation Between PNH Clone Size in BM Cells and PB Neutrophils

The PNH clone size measured in PB neutrophils correlated well with that of BM mature neutrophils, monocytes, and NRBCs (Figure 7A, Figure 7B, and Figure 7C). As mentioned above, the PNH clone size among mature lymphocytes (largest in the NK cells, followed by B and T cells) was smaller than among other WBCs and NRBCs, as shown in Figure 7D. Plasma cells showed very small or absent PNH cells (Figure 7D). As shown in Figures 7A-7C, some cases demonstrated a relatively larger PNH clone size among neutrophils, monocytes, and NRBCs in BM than in PB neutrophils. These were mostly seen in patients with significant PB neutropenia.

Detection of PNH Cells in BM Analyzed With Antibodies Used for Routine Diagnostic Purposes

Antibodies recognizing CD45, CD11b, CD13, CD33, CD64, CD16, and CD14 are routinely used in the evaluation of BM by FC in patients with a variety of hematologic conditions. To detect PNH cells using these reagents,

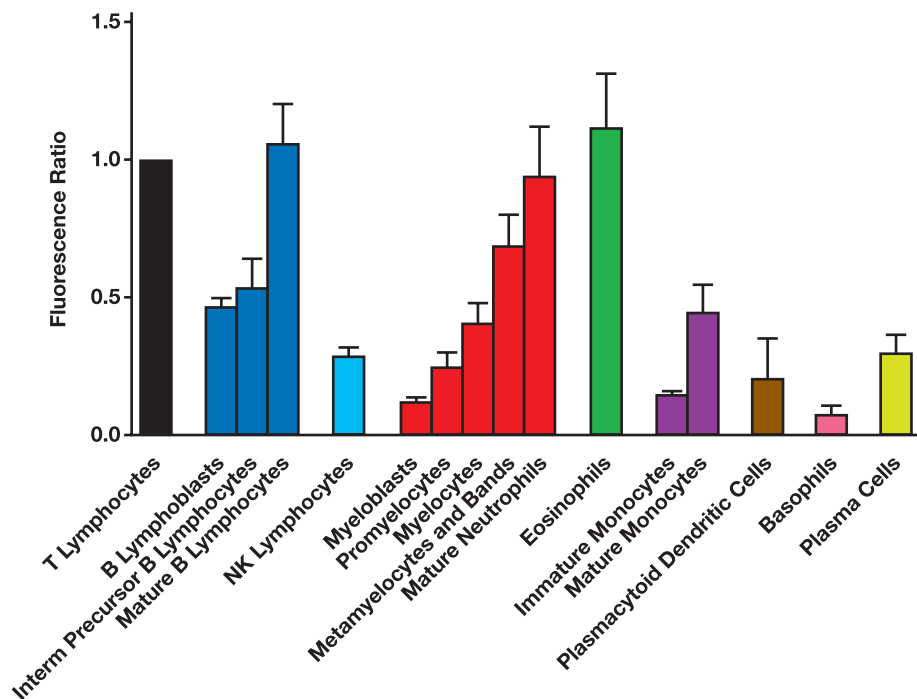


Figure 4 Fluorescence from bone marrow cells stained with fluorescently labeled aerolysin (FLAER) in 11 healthy volunteers. With cell maturation, there is progressive fluorescence intensity in B cells, neutrophils, and monocytes. Basophils, plasmacytoid dendritic cells, and plasma cells display relatively low fluorescence. The fluorescence was measured using a ratio between the mean fluorescence intensity of the population of interest and that of T lymphocytes from the same sample. Error bars represent a standard deviation. Interm, intermediate.

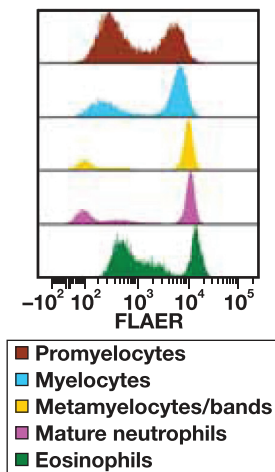


Figure 5 Histograms showing fluorescently labeled aerolysin (FLAER) binding to granulocytes in bone marrow cells from a patient with paroxysmal nocturnal hemoglobinuria (PNH). All granulocytic subpopulations demonstrate the presence of normal cells (binding FLAER) and abnormal, PNH-type cells with reduced or no FLAER binding (glycophosphatidylinositol [GPI]-negative cells). In this case, more than one GPI-anchored protein defective cell subpopulation is seen (best demonstrated among mature granulocytes).

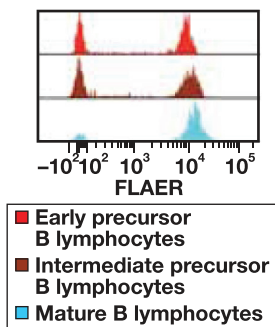


Figure 6 Histograms showing fluorescently labeled aerolysin (FLAER) binding to B lymphocytes in bone marrow cells from a patient with paroxysmal nocturnal hemoglobinuria (PNH). All B-cell subpopulations demonstrate the presence of normal cells (binding FLAER) and abnormal, PNH-type cells with reduced or no FLAER binding (glycophosphatidylinositol-negative cells).

neutrophils were analyzed independently of eosinophils based on the intense CD45 expression, high SSC signals, and low/absent expression of CD16 by eosinophils **Figure 8A**. Then, the most mature neutrophils were selected based on their intense CD11b and CD13 expression **Figure 8B**. In the BM of patients with PNH, examination of CD16 (a GPI-anchored protein) expression on the most mature neutrophils demonstrated discrete cell populations with reduced expression of CD16, clearly

separated from their normal counterparts **Figure 8C**. Similarly, in contrast to monocytes from healthy individuals **Figure 9A**, BM monocytes from patients with PNH demonstrated low expression of CD14 (also a GPI-AP) while CD45 expression was as intense as in normal mature monocytes **Figure 9B**. Normal immature monocytes also showed poor CD14 expression, but these cells demonstrated dimmer CD45 expression than PNH cells. When FLAER was added to the antibody panel, we showed that the abnormal neutrophils and monocytes detected in the BM of patients with PNH as described above did not bind FLAER, confirming that these were PNH cells **Figure 9C**. The fraction of mature neutrophils with low or absent CD16 expression correlated well with the PNH clone size detected in circulating neutrophils **Figure 10**. Although the PNH monocytes could be visually identified, they overlapped somewhat with early normal monocytes, making their quantitation very difficult.

On blinded examination of granulocytes and monocytes as described above, only one (a case of hypereosinophilia) of the 105 marrows from patients without detectable PNH cells in PB demonstrated abnormal mature neutrophils mimicking PNH (expressing dim or no CD16). BM monocytes in this case, however, did not show abnormalities. Only 90 of those 105 marrows had sufficient monocyte numbers to be analyzed. Only one of these 90 marrows (a case of acute myelomonocytic leukemia) had monocytic changes simulating PNH (expressing intense CD45 and lacking CD14 expression). In this case, neutrophils were immunophenotypically normal. Only in one of the 50 marrows (a case with severe neutropenia) from patients with detectable ($\geq 1\%$) PNH neutrophils in the PB was the PNH clone not detectable in BM neutrophils. However, BM monocytes in this case demonstrated the CD14 deficiency typical of PNH. False PNH-negative cases based on monocytic assessment were more frequent (6 of 50), but all of these cases showed neutrophil changes consistent with PNH. Thus, in our cases, the detection of PNH cells in the BM based on the combined analysis of granulocytic and monocytic changes using routine reagents reached a sensitivity and specificity of 100%.

Discussion

Few studies have been performed delineating the patterns of expression of GPI-AP in different hematopoietic cell lineages in the BM and during their differentiation,^{21,22} and very limited information is available on the use of FLAER in BM cells of patients with PNH.¹⁵ In our study, we performed a comprehensive FC analysis of BM cells using a large array of antibodies in conjunction with

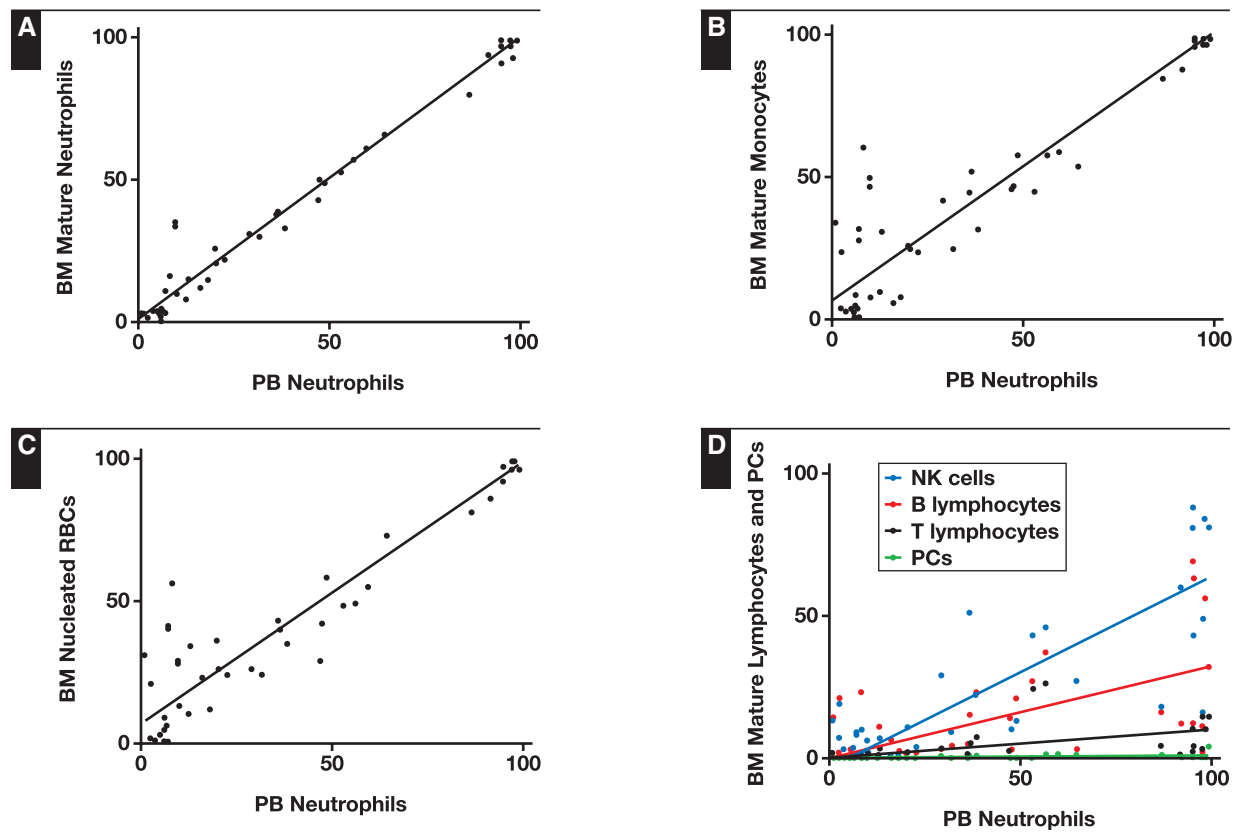


Figure 7 Correlation between paroxysmal nocturnal hemoglobinuria (PNH) clone size measured in peripheral blood (PB) neutrophils and on different bone marrow (BM) cell populations ($n = 47$): **(A)** mature neutrophils, **(B)** mature monocytes, **(C)** nucleated RBCs, and **(D)** other cells, including natural killer (NK) cells, mature B and T lymphocytes, and plasma cells (PCs). Detection of PNH in BM cells was based on fluorescently labeled aerolysin binding to WBCs and on CD55/CD59 expression by nucleated RBCs. **A**, $r = 0.99$; **B**, $r = 0.92$; **C**, $r = 0.93$.

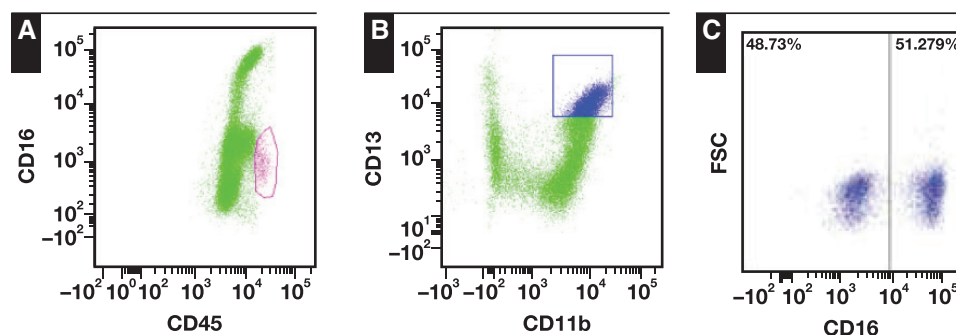


Figure 8 Gating strategy for detection of paroxysmal nocturnal hemoglobinuria (PNH) cells among mature neutrophils in bone marrow, based on commonly used antibodies. Granulocytes are first selected based on their CD45 expression and side light-scatter properties (not shown). **A**, Among granulocytes, neutrophils (green) and eosinophils (pink) are distinguished based on CD45 and CD16 expression. **B**, Mature neutrophils (blue) can then be identified based on their intense coexpression of CD11b and CD13. **C**, PNH-type mature neutrophils are identified based on their decreased or absent CD16 expression.

FLAER to define cell lineages and cell differentiation stages in the BM of healthy volunteers and in patients with acquired BM failure and PNH.

We first showed the distribution and patterns of FLAER binding to normal BM cell populations and

confirmed that virtually all cells bind this reagent. The expression of GPI-AP in normal BM and blood cells has been previously shown using antibodies against CD14, CD16, CD24, CD48, CD52, CD58, CD59, CD66b, CD87, CD109, and CD157,²¹ but for nonerythroid cells,

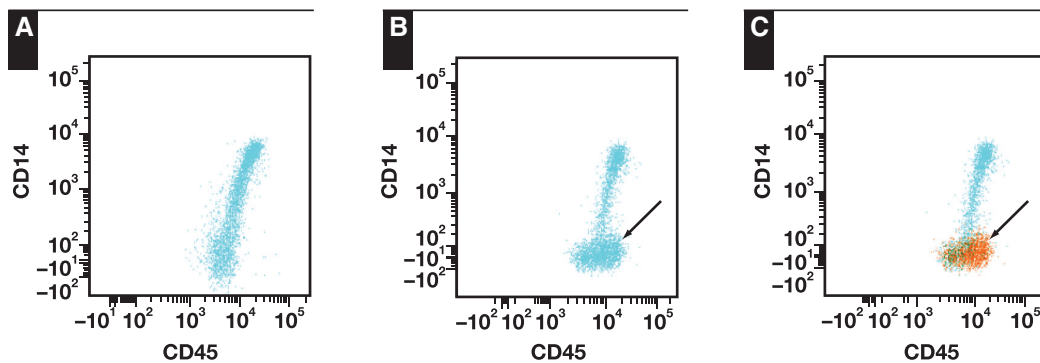


Figure 9 Detection of paroxysmal nocturnal hemoglobinuria (PNH) cells among mature monocytes in bone marrow (BM), based on commonly used antibodies. Monocytes are first selected based on their bright CD64 (or CD33) expression and side light-scatter properties (not shown). **A**, Dot plot of monocytes in BM of a healthy subject showing expression of CD14 and CD45. As monocytes mature, CD14 is increasingly expressed. **B**, Dot plots of monocytes in BM of a patient with PNH showing a cell population with reduced or absent CD14 and preserved CD45 expression (arrow). **C**, For confirmation, in this case, fluorescently labeled aerolysin (FLAER) was used to select PNH monocytes, which are shown in orange (corresponding to FLAER-negative cells).

FLAER is a superior reagent because of its higher signal-to-noise ratio and resolution.¹⁴ Using this reagent, we showed that BM cells bind FLAER increasingly as they undergo differentiation and that the most mature elements show the highest level of FLAER binding. Of interest, early neutrophils, including myeloblasts, demonstrated a bimodal or multimodal distribution of FLAER binding suggesting GPI heterogeneity among these cells, a feature not observed among early B lymphocytes.

We then applied a similar analysis to patients with detectable PNH clones in PB (mostly patients with AA

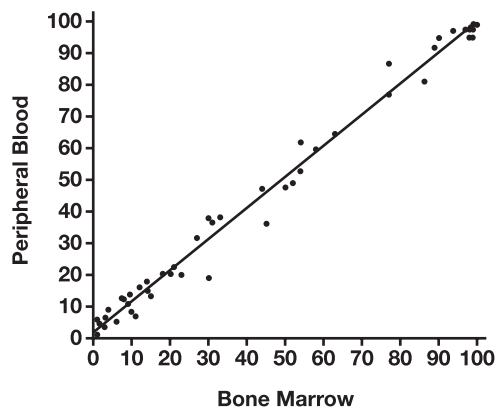


Figure 10 Correlation between paroxysmal nocturnal hemoglobinuria (PNH) clone size detected in bone marrow (BM) mature neutrophils based on commonly used antibodies and PNH neutrophils in peripheral blood enumerated based on fluorescently labeled aerolysin ($n = 50$). The detection of PNH in mature BM neutrophils was based on decreased or absent CD16 expression, as demonstrated in Figure 8. $r = 0.99$.

or MDS). In these patients, we showed the presence of abnormal cells with deficient FLAER binding in all BM lineages studied. However, similar to previous observations,²³ PNH clones were larger in granulocytes, monocytes, and erythroid precursors than in mature lymphocytes, supporting the notion that most of the cells of lymphoid lineage are long-lived, predating the emergence of PNH clones.²⁴ Also, variable clone sizes were observed among different types of lymphocytes: in most cases, PNH clone size was largest in NK cells followed by B and T cells. Plasma cells, being long-lived, showed extremely few or no GPI-deficient cells. Detection of PNH cells was thus possible in most BM cells and progenitors. However, a clear distinction between early normal myeloid elements and PNH neutrophils was not always possible due to poor FLAER binding shown by both, resulting in overlapping peaks on the fluorescence histograms.

The detection of PNH clones and the accurate assessment of PNH clone size are important, particularly in patients in whom clones increase to a level of clinically significant disease, which is characterized by intravascular hemolysis and/or thrombosis. We demonstrated that the PNH clone size measured in BM mature granulocytes and monocytes using FLAER and even in erythroid precursors (using CD55/CD59) is comparable to that of PB neutrophils, making BM aspirates suitable for PNH detection and quantitation. PNH analysis in BM should be most valuable in patients with neutropenia, a common finding in patients with BM failure, in whom acquiring a sufficient number of neutrophils from PB may be difficult or not possible. Also, in these patients, circulating RBCs may be affected by hemolysis and/or transfusions, leading to inaccurate PNH quantitation. Infrequent cases

in our study showed a higher number of GPI-defective erythroid precursors and monocytes than that of mature neutrophils. The reason for this discrepancy is unclear, although we have observed in some of these patients who have been studied longitudinally that the low neutrophil PNH clone size represents a transient phenomenon (data not shown). Quantitating PNH lymphocytes is possible but since clone sizes among these cells are smaller than those of neutrophils or NRBCs, this information would be of little clinical or therapeutic significance.

A limitation of this study was that the analysis was only aimed at detecting 1% or more PNH. This may be relevant since some studies have suggested that the presence of even minor PNH populations in patients with AA predicts response to immunosuppressive therapy.^{25,26} However, other studies, including one from our own institution, did not confirm this finding.^{27,28} Nevertheless, it may be useful in the future to determine the limit of detection of PNH clone size in the BM below 1%. This assessment was beyond the scope of our current study.

Antibodies recognizing CD45, CD11b, CD13, CD33, CD64, CD16, and CD14 are commonly used in the flow cytometric evaluation of BM in patients with a variety of hematologic conditions. We showed that BM aspirate analysis performed by FC with a combination of these antibodies can identify mature granulocytes and monocytes (most likely representing blood elements admixed to BM cells in the aspirates²⁹), with reduced expression of CD16 and CD14 (GPI-AP), respectively, that are indicative of PNH. This analysis (without the use of FLAER or CD55/CD59) could be easily incorporated into routine testing of marrow cells by FC and serves as a first step in a diagnostic algorithm for PNH detection, in which additional markers such as FLAER, CD55, and CD59 can confirm the presence of GPI-defective cells. Because of the simplicity of the assay and the easiness of obtaining sufficient cellular material, PB remains the sample of choice for PNH testing. However, our data show that an appropriate analysis of BM could yield valuable information in the detection of PNH. As others suggested,¹⁶ this would be particularly useful in patients whose BM samples are submitted to flow cytometry laboratories to evaluate hematologic disorders and in whom the presence of PNH clones is not suspected.

Although individually, mature neutrophils and monocytes can reveal abnormalities of CD16 and CD14 expression highly suggestive of PNH, these changes may be observed in other conditions leading to false interpretations. For example, lack of CD16 expression on neutrophils may reflect a polymorphism due to the absence of the Fc γ receptor IIIb gene.³⁰ Phenotypic changes mimicking those observed in PNH may also be seen in MDS, in which antigen expression may be altered as a consequence

of distorted maturation.³¹ However, the expression pattern of other informative markers helps distinguish PNH cells from dysplastic cells.³²⁻³⁴ Also, marked eosinophilia or severe mature neutropenia can affect the interpretation of neutrophil abnormalities mimicking PNH. Similarly, some leukemic monocytes may resemble PNH cells, and a reduced number of monocytes may preclude an adequate analysis or reduce sensitivity of detection. However, when both granulocytes and monocytes are analyzed, detection of true PNH cells using routine antibodies should be possible in virtually all cases with 1% or more PNH clone size.

Studying the expression of GPI-APs in different cell lineages and various stages of cell differentiation should enhance our understanding of the pathogenesis of PNH. Also, indirectly, since the GPI-AP abnormalities occur as a consequence of a somatic mutation in stem cells, these studies should shed light on the life span of hematopoietic and lymphoid cells, including infrequent lineages such as dendritic cells, basophils, or NK cells. As somatic mutations involved in myeloid neoplasms have been observed in cells bearing *PIG-A* mutations,³³ it may be of interest to evaluate GPI deficiencies in the BM of patients with myeloid disorders to assess clonal architecture and evolution in these conditions.

In conclusion, although PB is currently considered the only valid source for the laboratory diagnosis of PNH,³⁵ our observations demonstrate that BM aspirate samples are equally valuable for the detection and quantification of PNH cells.

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