Original Articles

Diagnosing PNH with FLAER and Multiparameter Flow Cytometry

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Background: PNH is an acquired hematopoietic stem cell disorder leading to a partial or absolute deficiency of all glycophosphatidyl-inositol (GPI)-linked proteins. The classical approach to diagnosis of PNH by cytometry involves the loss of at least two GPI-linked antigens on RBCs and neutrophils. While flow assays are more sensitive and specific than complement-mediated lysis or the Hams test, they suffer from several drawbacks. Bacterial aerolysin binds to the GPI moiety of cell surface GPI-linked molecules and causes lysis of normal but not GPI-deficient PNH cells. FLAER is an Alexa488-labeled inactive variant of aerolysin that does not cause lysis of cells. Our goals were to develop a FLAER-based assay to diagnose and monitor patients with PNH and to improve detection of minor populations of PNH clones in other hematologic disorders.

Methods: In a single tube assay, we combined FLAER with CD45, CD33, and CD14 allowing the simultaneous analysis of FLAER and the GPI-linked CD14 structure on neutrophil and monocyte lineages.

Results: Comparison to standard CD55 and CD59 analysis showed excellent agreement. Because of the higher signal to noise ratio, the method shows increased sensitivity in our hands over single (CD55 or CD59) parameter analysis. Using this assay, we were able to detect as few as 1% PNH monocytes and neutrophils in aplastic anemia, that were otherwise undetectable using CD55 and CD59 on RBC's. We also observed abnormal FLAER staining of blast populations in acute leukemia. In these cases, the neutrophils stained normally with FLAER, while the gated CD33bright cells failed to express normal levels of CD14 and additionally showed aberrant CD45 staining and bound lower levels of FLAER.

Conclusion: FLAER combined with multiparameter flow cytometry offers an improved assay for diagnosis and monitoring of PNH clones and may have utility in detection of unsuspected myeloproliferative disorders. © 2007 Clinical Cytometry Society

Key terms: FLAER; PNH; multiparameter flow cytometry; hematologic abnormalities

Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired hematopoietic stem cell disease (1,2) that is caused by a somatic mutation of the X-linked phosphatidyl-inositol glycan (*PIG-A*) gene (3,4). Since the PIG-A protein is involved in the initial stage of synthesis of the glycosylphosphatidyl-inositol (GPI) anchor (5,6), these defects result in partial or absolute deficiency of all GPI-linked proteins/glycoproteins in a clone of hematopoietic stem cells (7–9). Expansion of PNH-like clones can also be detected in cases of aplastic/hypoplastic/myelodysplastic syndromes caused by an immune-mediated failure of normal hematopoiesis or defect in hematopoietic stem cells (10).

Typical clinical features of PNH are: bone marrow failure of variable severity, thrombosis in unusual sites, chronic intravascular hemolytic anemia that leads to hemoglobinuria, iron deficiency anemia, and increased inci-

dence of acute myeloid leukemia (11). Activated serum complement was shown to play an important role in the hemolytic anemia and two GPI-linked structures CD55 (Decay Accelerating Factor, DAF) and CD59 (Membrane Inhibitor of reactive lysis, MIRL) on red blood cells play an important role in the control of complement (12,13). While the absence or partial expression of CD55 and CD59 is specific for PNH, CD59 deficiency alone appears

to be responsible for hemolysis and other clinical symptoms of PNH (14,15).

Diagnosis and follow-up of PNH patients has been greatly improved by the advent of flow cytometry-based assays (16–18, reviewed in Refs. 19 and 20). Because of the historical role of red blood cells in the diagnosis of PNH (21), most flow cytometric approaches have involved the staining of patients' red blood cells with CD55 and CD59 (18,22). Flow cytometric analysis of RBCs in untransfused patients can be used to quantitate the Type III (complete deficiency), type II (partial deficiency), and type I (normal expression) clones (19,20). There are several potential problems with this approach; the lysis of Type III RBCs and or the presence of recently transfused normal RBCs significantly reduces the ability to detect PNH RBCs. As PNH is a rare disease, deficiency of at least two GPI-linked structures on more than one lineage, typically, neutrophils, is an additionally required criterion to establish a diagnosis. In situations of prior hemolysis and/or recent red cell transfusion, detection of PNH granulocytes best reflects the size of the PNH clone. However, severe neutropenia in some patients makes it difficult to identify the target population using light scatter characteristics alone. Furthermore, in a small number of PNH patients, only neutrophils are affected (18). Because of their prolonged life span, lymphocytes are not normally a good target population for analysis because only those arising post-disease onset will be deficient in GPI-linked structures; the majority will express GPI proteins whereas neutrophils, monocytes, and red cells do not.

Although a rare disease, the PNH assay is frequently requested and in the 2004 calendar year, we screened 141 samples for PNH and detected only three bona fide cases of PNH. At our institutions, we used the two-part Biocytex™ kit (Biocytex, Marseille, France), a quantitative immunocytometric approach that utilizes fluorescent beads to set windows of analysis and the staining of CD55 and CD59 on both red cells (Redquant™) and neutrophils (Cellquant™). The Biocytex assay is labor-intensive to set up, given the low frequency of the disease, and the Cellquant assay for neutrophils must be set up within 8 h of sample draw. Given the large number of referral samples that are analyzed at our institution, we were unable to meet the latter criterion on a number of samples.

An alternative approach that makes it possible to perform less extensive testing for the diagnosis of PNH has more recently been suggested that utilizes the bacterial toxin Aerolysin. Proaerolysin is a 52-kDa protein secreted by *Aeromonas hydrophila*. After proteolytic nicking at the C-terminus, the active form, Aerolysin is generated that binds to cell surface structures and oligomerizes, forming channels that result in cell lysis (23). Aerolysin does not lyse PNH cells and it was shown that the toxin bound to the GPI moiety of GPI-linked structures (24,25). Initially, this reagent was used to enrich rare GPI-negative PNH clones. Subsequently, a fluorochrome-conjugated (Alexa 488) version of a non-lysing, mutated

form of proaerolysin (FLAER) was generated that retained specificity for GPI-linked structures without causing cell lysis. FLAER is more sensitive than CD59 at detecting small abnormal granulocyte populations in patients to a level of approximately 0.5%. It was claimed that FLAER gave a more accurate assessment of the GPI anchor deficit in PNH (26). However, FLAER cannot be used to assess PNH clones in the erythrocyte lineage, since the latter do not possess surface-bound proteolytic enzymes needed to process the Proaerolysin. Further excluding the utility of aerolysin in the detection of GPI-linked structures on red cells is the observation that glycophorin binds weakly to proaerolysin despite the fact that this major red cell glycoprotein is not GPI-linked (27).

While simple flow-based FLAER assays for PNH have been developed (26,28), the ability to detect rare PNH clones in closely related diseases such as aplastic anemia and myelodysplasia would be enhanced by a more sophisticated multi-parameter approach. In this study, we developed such an approach based upon the staining of a single pre-lysed blood sample with antibodies to CD14 (GPI-linked), CD33 (non-GPI-linked), CD45, and FLAER. In comparison with our predicate method, the assay is more sensitive, less expensive, and takes only 40 min from sample draw. Interestingly, we have also identified aberrant FLAER expression in several cases of undiagnosed acute myeloid leukemia, sent initially for PNH testing.

MATERIALS AND METHODS
FLAER Assay

CD33PE, CD45ECD, and CD14PE-Cy5 were purchased from Beckman Coulter, (Miami, Florida). Fluorescent Aerolysin (FLAER) was purchased from Protox Biotech (catalogue number FL2, Victoria, British Columbia, Canada; www.protoxbiotech.com). The FLAER was dissolved in 1 mL of Phosphate Buffered Saline (PBS) and "stock" aliquots of 50 μL stored at −20°C. "Working" aliquots were diluted 1:10 with PBS and stored at 4°C and used within 1 week.

Sample Preparation

Red blood cells were lysed by addition of 0.5 mL of Optilyse C (Beckman-Coulter) to 100 μL of anti-coagulated (EDTA) peripheral blood. After 10 min at 20°C, 1 mL PBS was added and the suspension incubated for another 10 min. The suspension was centrifuged at 400g for 5 min, the supernatant decanted and the pellet resuspended in 100 μL of PBS/2% albumin. CD33PE (10 μL), 5 μL CD45ECD, 5 μL CD14PE-Cy5, and 10 μL of FLAER "working" solution was added to each tube and incubated for 15 min at 20°C. Finally, 1 mL of PBS was added and the sample analyzed on an FC500 cytometer (Beckman Coulter).

Biocytex Kits

Biocytex (Marseille, France) provide a two-part, seven-tube assay kit to detect PNH clones without the use of control (non-PNH/normal) cells.

Cellquant™. In this kit, the alpha and beta beads are pooled in one tube from the manufacturer and the CD55- and CD59-negative regions are established from the analysis of the pooled beads. Red blood cells are first lysed by incubation of 400 µL of whole blood with 8 mL of lysing reagent (1 : 10 freshly made from stock supplied). After 12 min at room temperature, the blood sample is centrifuged for 5 min at 300g. After discarding the supernatant, the white cell pellet is suspended in 150 µL of reagent diluent as for Redquant. Three tubes are set up, the first of which contains the pooled alpha/beta beads. Tubes 2 and 3 contain 50 µL of lysed blood sample. The three tubes are stained and analyzed in a similar manner as described below for red cell analysis, except that granulocytes are identified and gated by linear forward versus linear side scatter. Cellquant can be used to analyze samples up to 8 h post sample draw. An example of PNH granulocytes analyzed by this methodology is shown in Figure 1, (bottom row).

Redquant™. This kit contains pre-calibrated fluorescent beads for CD55 and CD59 expression to establish threshold fluorescence values against which staining of sample red blood cells by CD55 and CD59 can be compared. Four tubes are set up, the first two of which contain 40 µL of "alpha" beads (to establish CD55 expression), or "beta" beads (to establish CD59 expression) respectively. Ten microliter of blood sample is diluted with 1.5 mL of kit diluent. Twenty microliter aliquots of the diluted blood are stained with either 20 µL of CD55 (tube 3) or 20 µL of CD59 (tube 4). After 12 min at room temperature, 20 µL of anti-mouse IgG FITC is added to all 4 tubes. After 12 min incubation, 2 mL of diluent is added to each tube. Windows of analysis for CD59- and CD55-stained red cells are established by acquiring the beta and alpha beads according to the manufacturer's instructions. The acquisition and analysis of the red cells stained with CD55 or CD59 is performed on gated red cells (linear forward versus log side scatter)
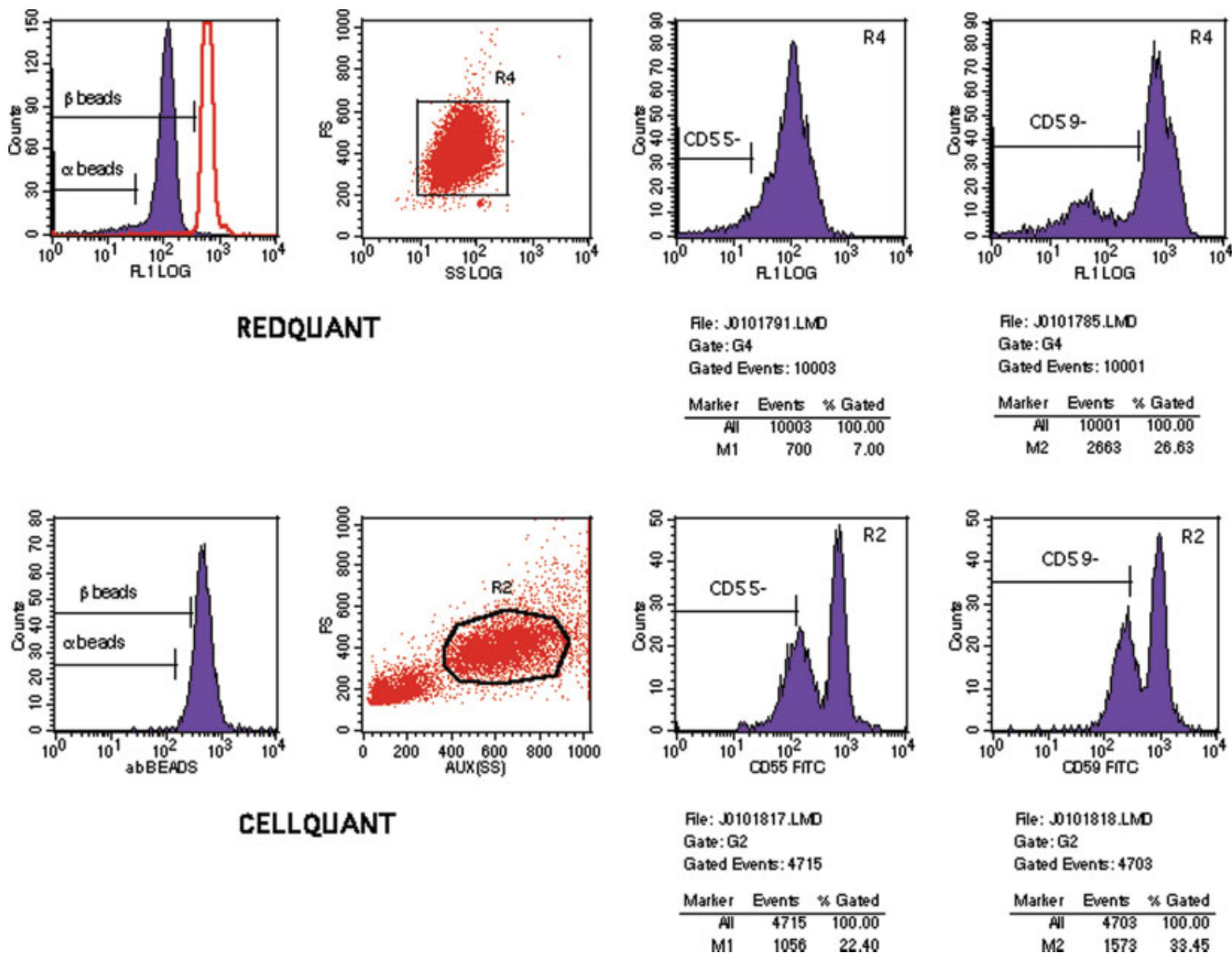

FIG. 1. Analysis of fresh PNH sample with Biocytex Redquant™ and Cellquant™ Kits. Gating regions for CD55− and CD59− events were established using the alpha and beta beads respectively. Neutrophils were gated into region R2 and assessed for CD5 and CD59 staining as described in Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
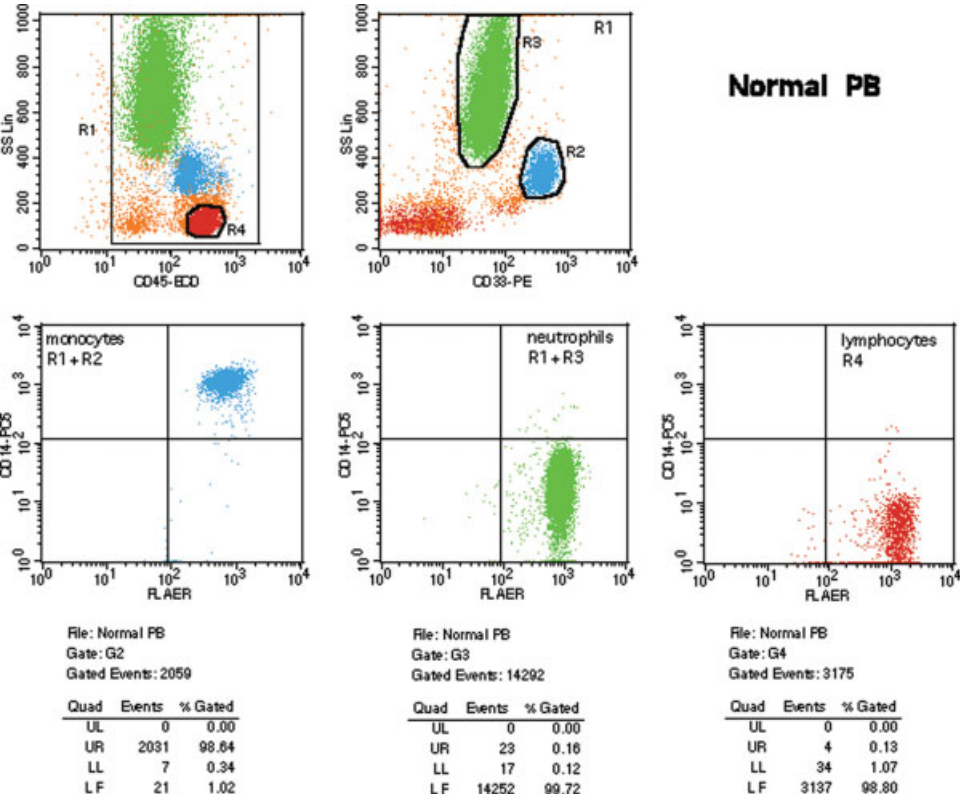
FIG. 2. Normal peripheral blood sample stained with FLAER, CD33, CD45, and CD14. Cells gated in Region R2 exhibit the composite phenotype of normal monocytes (CD45+, CD33bright, CD14+, and FLAER+). Cells gated in region R3 exhibit the composite phenotype of neutrophils (CD45lo, CD33lo, CD14lo, and FLAER+). Cells gated in Region R4 exhibit the composite phenotype of lymphocytes (CD45hi, CD33−, CD14−, and FLAER+). Less than 0.5% monocytes and neutrophils display a CD14 negative, FLAER negative PNH phenotype.

FIG. 3. Fresh PNH sample stained with FLAER, CD33, CD45, and CD14. Monocytes (71%) from R2 exhibit a CD14-, FLAER-PNH phenotype (lower left). Neutrophils (51%) from R3 exhibit a CD14-, FLAER- PNH phenotype (lower middle).

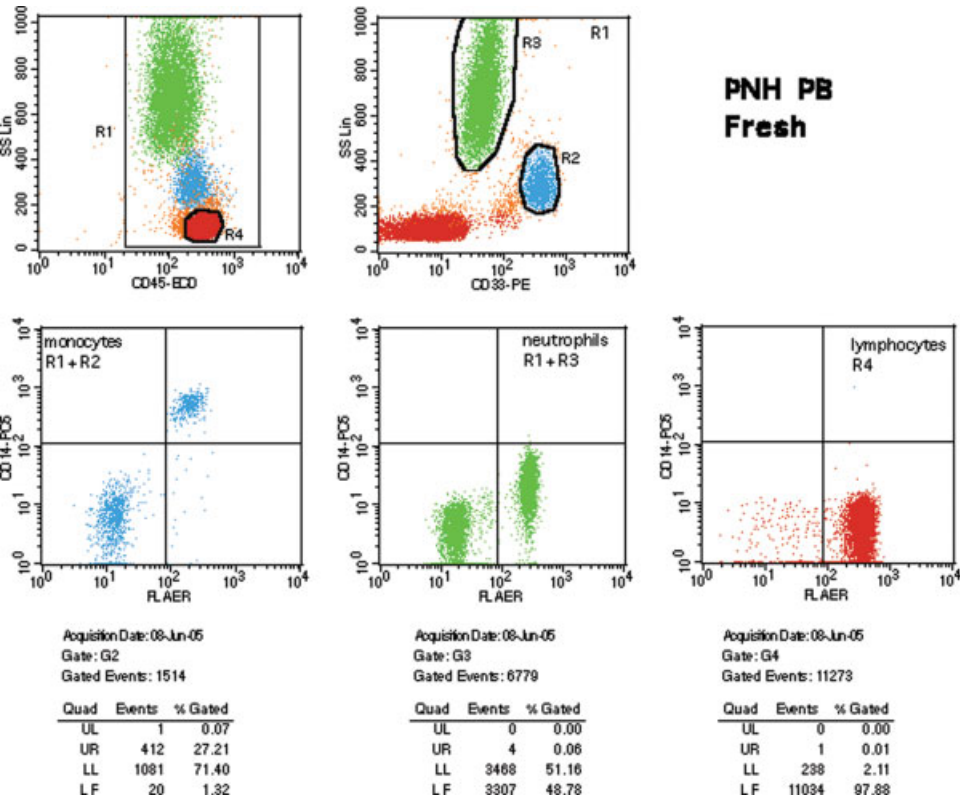using the same gating regions established using the calibrator beads. For a normal sample that shows no deficiency in CD55 and CD59 expression, the analysis regions should not contain more than 3% of the gated cells. Redquant can be used to analyze samples up to 24 h post sample draw. An example of PNH red cells analyzed by this methodology is shown in Figure 1, top row.

CD59 Assay for Red Blood Cells

Ten microliter of whole blood was diluted with 1.5 mL of PBS and 20 μL added to each of two tubes. Twenty microliter of CD59 FITC was added to the second tube, the first serving as an unstained control. After 15 min at room temperature, the cells were washed by centrifugation at 1,500g for 5 min. The red cell pellets were resuspended in 1 mL of PBS and 10,000 red blood cells, identified by light scatter (log or linear forward versus log side scatter) acquired in listmode.

RESULTS
Analysis of PNH Patient Sample with Biocytex Cellquant™ and Redquant™ Kits

Figure 1 shows the analysis of a PNH sample with the Redquant (top row) and Cellquant (bottom row) kits. For the Redquant assay alpha (filled histogram) and beta (open histogram) gating regions were set according to the fluorescence of the respective alpha and beta beads. Analysis of the patient's red blood cells using the gating regions specified by the beads as per manufacturer's recommendations indicated the presence of 7% CD55− and 26.6% CD59− PNH red cells. With the Cellquant assay, 22.4% CD55− and 33.5% CD59− cells were detected. By Biocytex criteria, the diagnosis of PNH requires at least three of the four parameters to identify > 3% negative cells. Thus, this sample was unequivocally shown to contain PNH clones.

Examination of the histograms shown for CD55 and CD59 staining of granulocytes (Cellquant bottom row) and red cells (Redquant top row) shows the automatic gate settings to be sub-optimal in terms of accurately quantifying the number of PNH clones. To better estimate the PNH clone size, it is possible to manually optimize the gate setting for CD55 and CD59 staining of granulocytes, and the CD55 and CD59 staining of red cells. When manual gating was applied, CD55 and CD59 detected approximately 45 and 44% PNH granulocytes respectively, while CD59 detected 19% PNH red cells (data not shown). Given that CD55− red cells did not separate adequately from CD55+ red cells, further optimization of this gate setting was not attempted.

Analysis of Normal and PNH Samples Using FLAER, CD45, CD33, CD14, and Multiparameter Analysis

Figure 2, shows an example of the gating strategy utilized in the analysis of a normal peripheral blood sample stained with CD33PE, CD45ECD, CD14PE-Cy5, and FLAER. Gating region R1 was established on plot 1 to include all nucleated white blood cells and exclude any debris remaining after the red cell lysis step. A gating

region R4 was also established to include only lymphocytes with bright CD45 staining and low side scatter. The CD45+ events were next displayed on a CD33 versus side scatter plot (plot 2). Two more gates were established to include monocytes in region R2 and neutrophils in region R3. Finally, monocytes (from regions R1 and R2), neutrophils (from regions R1 and R3), and lymphocytes (from region R4) were individually analyzed for CD14 versus FLAER expression (plots 3 through 5 respectively). Normal monocytes (plot 3) exhibit bright CD14 and bright FLAER staining, whereas normal neutrophils (plot 4) exhibit only weak CD14 staining with bright FLAER staining. Lymphocytes (plot 5) are unstained by CD14 while staining brightly with FLAER.

Figure 3 shows an example of a PNH sample (AS) analyzed with the same gating strategy as used for the normal sample. This PNH sample was identical to that used above (Fig. 1) in the Biocytex analysis. As shown in plot 3, monocytes gated from region R2 formed two separate clusters on CD14 versus FLAER analysis. While some apparently "normal" monocytes were detected with characteristic CD14 and FLAER staining (upper right quadrant), about 71% of the monocytes were unstained by either reagent (lower left quadrant). Neutrophils from region R3 also formed two approximately equal well-defined clusters appearing as normal (CD14 weak/FLAER positive) cells, with a second population (51%) that was unstained by CD14 and FLAER (lower left quadrant). About 98% of the lymphocytes (region R4) from this patient sample were stained by FLAER and thus exhibit a normal (non-PNH) phenotype.

In several other cases analyzed with this methodology, we generally observed that the numbers of PNH monocyte and PNH granulocyte clones were closely similar in each patient sample (Table 1). Additionally, in one case, (VT) we were able to perform the FLAER test at two separate time points and in each analysis, the numbers of PNH monocyte and PNH granulocyte clones remained constant at about 84 and 91% respectively over a 7-month period. In contrast, the numbers of detectable PNH lymphocyte clones increased from about 12% to over 16% (Table 1).

Assay Stability

To determine the reproducibility of the FLAER assay over a 24-h period, the same patient sample (AS) was stained 24 h post sample draw. The sample was maintained on the bench at room temperature during this time. As shown in Table 1, very similar numbers of PNH monocyte clones were detected on the 24 h-old sample as were detected on the fresh sample. Similarly, the numbers of PNH granulocytes detected in the fresh and 24 h-old samples were the same.

We also performed analysis of a number of non-PNH samples at 1, 24, and 48 h post sample draw. No evidence of PNH clones was detected in over 85 normal samples analyzed at any time point (data not shown). Additionally we tested 2 PNH samples (DC and VT) that contained numbers of PNH clones in the 85–92% range at 1, 24, and 48 h post draw. In these cases at least, there was no significant diminution in the numbers of

Table 1
Enumeration of PNH granulocytes and monocytes with FLAER

Patient	Date d/m/y	FLAER (%) Monocytes	Granulocytes	Lymphocytes	CD59 (%) Type III/II
VT	29/10/04	83.7	90.9	11.7	ND
VB	07/12/04	97.5	95.23	2.7	ND
JW***	05/01/05	11.9	10.4	1.5	ND
AL**	28/02/05	11.3	11.4	1.1	ND
DC*	4/04/05	87.0	88.1	1.5	ND
DC*	5/04/05	85.2	86.9	1.5	ND
DC*	6/04/05	82.5	83.7	1.5	ND
AS*	8/06/05	71.4	51.3	2.0	ND
AS*	9/06/05	70.1	51.3	1.6	ND
DC	13/06/05	94.5	92.7	1.0	ND
AL**	30/06/05	7.3	7.1	1.4	ND
VT*	18/07/05	85.3	91.2	16.0	22.1/21.6
VT*	19/07/05	81.9	90.3	14.2	23.9/
VT*	20/07/05	84.4	90.3	14.2	27.8/
2808	21/07/05	95.9	93.4	2.1	29.1/<1
2809	21/07/05	14.8	14.4	2.1	6.0/<1
JD*	27/07/05	12.5	7.8	0.5	1.8/<1
JD*	28/07/05	8.6	8.2	0.5	ND
JD	18/08/05	12.0	7.2	1.0	ND
AL**	24/08/05	5.9	4.5	3.0	ND
PL	26/10/05	5.3	4.8	13.8	1.6/0.4
RB	8/11/05	95.0	95.5	7.00	ND
PL	16/11/05	5.2	4.5	22.5	0.4/2.1
VT	16/01/06	82.8	89.6	15.6	21.4/13.0
AL**	15/02/06	1.2	1.5	2.2	<1/1.0
MM	15/02/06	54.6	59.6	0.9	18.1/2.2
GF	07/03/06	62.0	61.4	15.1	10.4/0.7
DT	29/03/06	73.1	74.8	3.5	18.9/43.3
BS**	30/03/06	37.7	30.8	1.7	5.1/5.8
LH	13/04/06	94.4	93.4	13.1	<1/81.4

Patient samples labeled with * were tested over 24 and in some cases 48 h post sample draw.
Samples labeled with ** were initially diagnosed as aplastic anemia.
Patient sample labeled with *** was initially diagnosed with myelodysplastic syndrome.
All other samples were diagnosed as PNH.
ND = Not Done.
Type III/II indicates the ratio of Type III RBCs to Type II RBCs.

detectable monocyte and granulocyte clones over the 48 h time course (Table 1).

Assay Sensitivity

We have detected PNH clones in the 5–12% range in several patient samples (JW, AL, JD, and PL) at different time points using the FLAER assay. Similar numbers of PNH monocyte versus granulocyte clones were found in each case (Table 1). Patient PL had previously been diagnosed as PNH in August 2004 using the Biocytex kits (prior to the development of the FLAER assay). At that time approximately 52 and 57% of the granulocytes were negative for CD55 and CD59 respectively while only CD59 identified PNH red cell clones above the 3% threshold. Patients AL and JD were previously diagnosed with aplastic anemia. In the case of AL, we were able to detect PNH monocytes and neutrophils down to 1.2 and 1.5% respectively in the most recent sample. In a case of MDS (JW), we observed PNH monocyte and granulocyte clones of 11.9 and 10% respectively using the multiparameter FLAER assay. Using more conventional methodology, a combination of CD45 (to gate neutrophils) and CD59, <7% CD59- cells were detectable (data not shown).

During the early phase of the FLAER assay development, we routinely stained a normal PB sample alongside the "suspected PNH" sample. In over 85 samples analyzed, we found neither PNH monocytes nor PNH neutrophils above the 0.5% level, as long as gating regions R2 (monocytes) and R3 (neutrophils) were accurately drawn (Fig. 2).

CD59 Staining of PNH Red Cells

While loss of the GPI-linked structures CD55 and CD59 from red blood cells alongside the loss of the same markers from granulocytes is used to diagnose PNH using the Redquant™ and Cellquant™ kits, more information regarding the severity of the disease can be obtained by more detailed analysis of PNH erythrocytes. Erythrocytes exhibiting a total loss of GPI-linked structures are called Type III, while those showing partial or no loss of CD59 are called Type II or Type I respectively. Figure 4 shows the analysis of patient VT's erythrocytes drawn on January 2006. Approximately 21.4 and 13.0% of the erythrocytes exhibited a Type III and Type II phenotype respectively. This compares with values of about
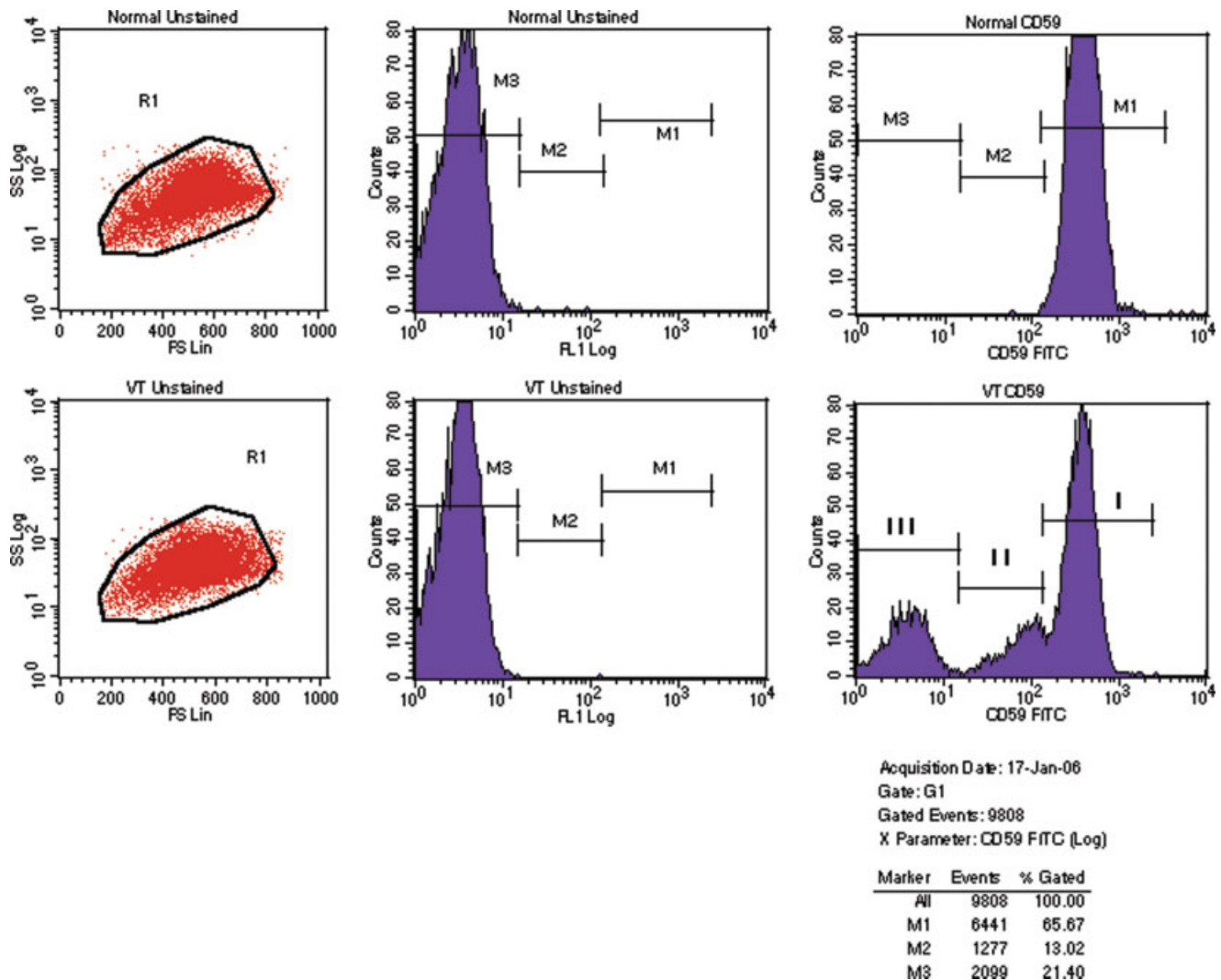
FIG. 4. CD59 staining of RBCs. Normal RBCs (top row) unstained (middle) or stained (right) with CD59FITC. RBCs from PNH patient VT (lower row) unstained (middle) or stained (right) with CD59FITC. Gating region M1 represents normal (type I) red blood cells. Cells in region M2 represent partially CD59 deficient (type II) PNH RBC clones. Cells in region M3 represent totally CD59 deficient (type III) PNH RBC clones. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

22% Type III and 21.6% Type II respectively on a sample drawn on July 2005 (Table 1), so indicating a relatively stable normal (Type I) red cell clone accounting for about 60–65% of erythrocytes in this non-transfused patient. In contrast, the number of FLAER-negative PNH monocyte and granulocyte clones was found to be consistently higher at about 84 and 90% respectively over a >1 year period of analysis (Table 1). This assay also appears to be robust and stable and we have not seen CD59-negative RBCs in over 100 samples that the FLAER assay determined to be "non-PNH".

Detection of Non-PNH Hematologic Abnormalities with FLAER Assay

During the course of this study, only a small number of peripheral blood samples tested showed the unequiv-ocal content of PNH clones, in keeping with the rare incidence of this disease. However, in several of the cases in which PNH could be readily excluded using the FLAER assay, the data generated suggested the presence of primitive cell populations. In the example shown in Figure 5, the neutrophils gated in region R3 showed a normal (non-PNH) phenotype with weak CD14 expression, intermediate CD33 expression, and normal FLAER staining. However, the bright CD33 stained cells in region R2 did not exhibit a normal monocyte phenotype with the majority of them expressing little or no CD14 together with weaker than normal FLAER staining. Back-scattering of these cells showed the majority of them to express low CD45 staining and exhibit low side scatter characteristics of immature blast-like cells. Also notable about this analysis was the large cluster of CD45-negative events remaining after red cell lysis. Subsequent analysis
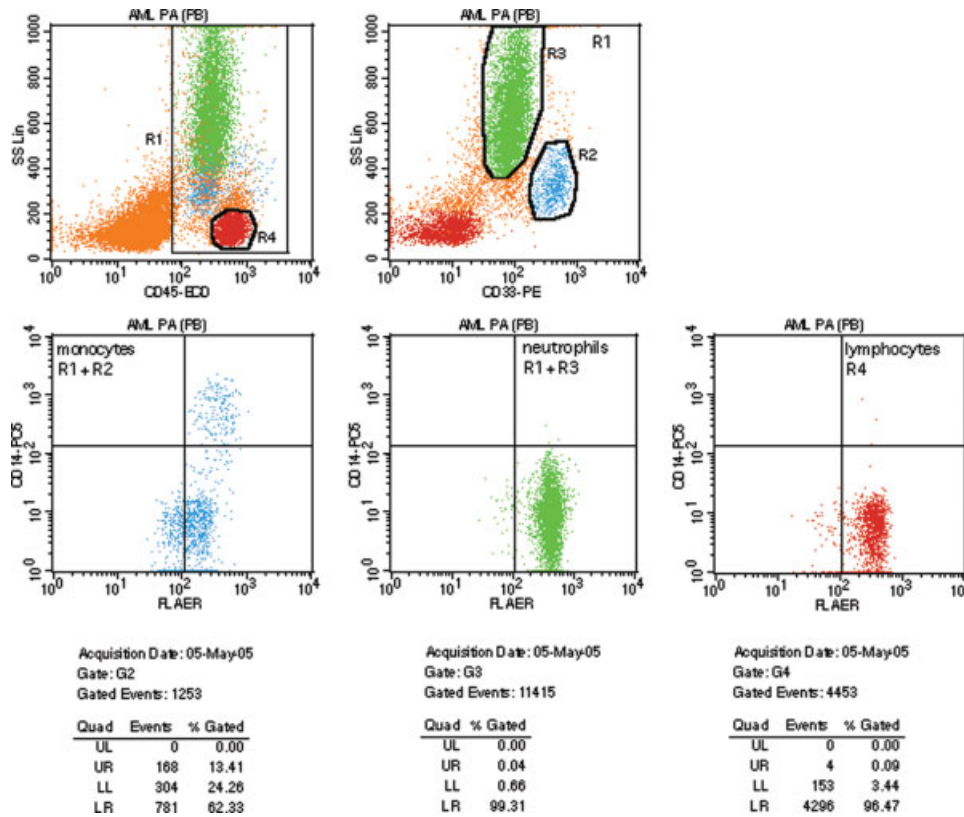
FIG. 5. Fresh sample sent for PNH testing stained with FLAER, CD33, CD45, and CD14. Neutrophils from R3 (lower middle) exhibited normal (non-PNH) phenotype. CD33+ cells from R2 (lower left) did not exhibit normal monocyte phenotype but instead showed weak/no CD14 staining and low CD45 staining characteristic of blast cells. Note the large cluster of CD45− nucleated red cells.

Acquisition Date: 05-May-05
Gate: G2
Gated Events: 1253

Quad	Events	% Gated
UL	0	0.00
UR	168	13.41
LL	304	24.26
LR	781	62.33

Acquisition Date: 05-May-05
Gate: G3
Gated Events: 11415

Quad	% Gated
UL	0.00
UR	0.04
LL	0.66
LR	99.31

Acquisition Date: 05-May-05
Gate: G4
Gated Events: 4453

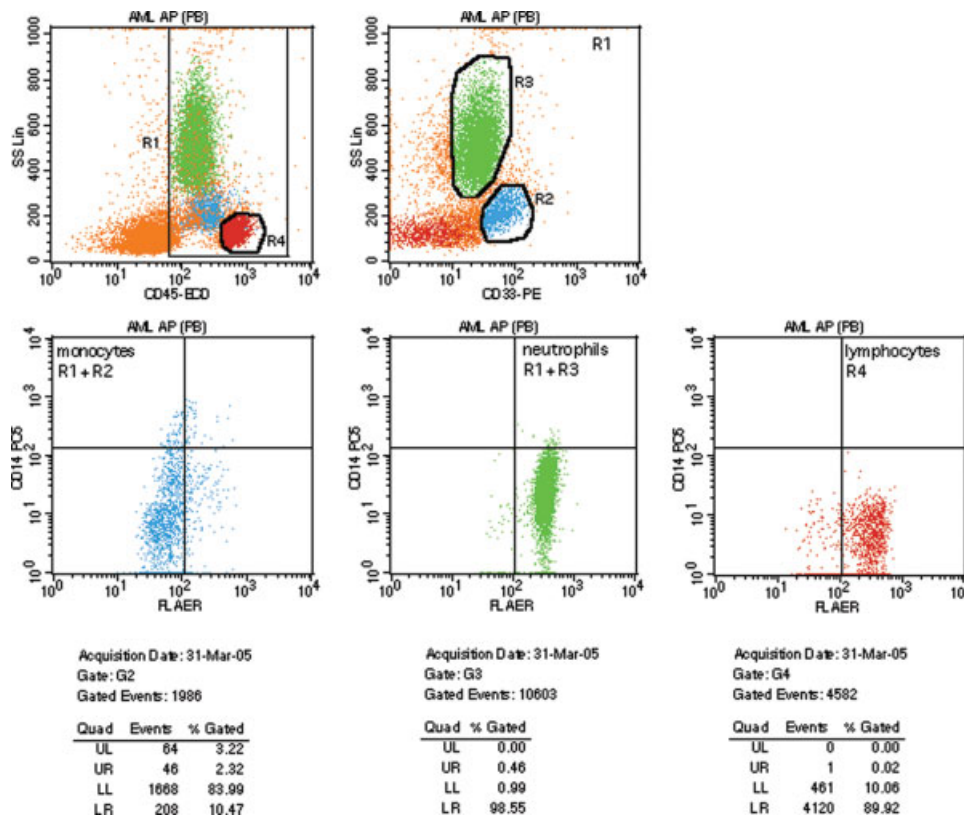| Quad	Events	% Gated
UL	0	0.00
UR	4	0.09
LL	153	3.44
LR	4296	96.47

FIG. 6. Fresh sample sent for PNH testing stained with FLAER, CD33, CD45, and CD14. Neutrophils from R3 exhibited normal (non-PNH) phenotype. CD33+ cells from R2 did not exhibit normal monocyte phenotype but instead showed weak/no CD14 staining and low CD45 staining characteristic of blast cells. Note the large cluster of CD45− nucleated red cells.

Acquisition Date: 31-Mar-05
Gate: G2
Gated Events: 1986

Quad	Events	% Gated
UL	64	3.22
UR	46	2.32
LL	1668	83.99
LR	208	10.47

Acquisition Date: 31-Mar-05
Gate: G3
Gated Events: 10603

Quad	% Gated
UL	0.00
UR	0.46
LL	0.99
LR	98.55

Acquisition Date: 31-Mar-05
Gate: G4
Gated Events: 4582

Quad	Events	% Gated
UL	0	0.00
UR	1	0.02
LL	461	10.06
LR	4120	89.92

of a blood smear from this sample revealed the presence of dysplastic, nucleated red blood cells, and a few blasts. Thereafter, a clinical diagnosis of Acute Myeloid Leukemia FAB type M6 was made on a marrow specimen.

In the second example shown in Figure 6, the neutrophils from region R3 again showed a normal expression of CD14, CD33, and FLAER, thereby ruling out PNH. In contrast, the CD33-stained putative monocyte population in region R2 was only weakly stained by FLAER and exhibited a very heterogeneous staining pattern with CD14. Back-scattering these events to CD45 versus side scatter revealed the majority of these cells to have an immature blast-like phenotype with low side scatter and low CD45 expression. Also apparent is the large cluster of CD45-negative events after erythrocyte lysis. A bone marrow aspirate was subsequently obtained and shown to contain 27% blasts based on CD45 versus side scatter analysis. These cells exhibited the composite phenotype of primitive myeloblasts and a diagnosis of Acute Myeloblastic Leukemia with multilineage dysplasia was subsequently made. Interestingly, a bone marrow biopsy performed on this patient only 1 month prior to the FLAER analysis had failed to reveal the presence of leukemic blasts.

We have additionally detected immature blast cells in several other samples submitted for "PNH testing", that upon further flow and morphologic analysis represented cases of bona fide leukemia, including a case of AML-M1, another case of myelomonocytic leukemia (AML-M4), and a case of CMML in putative blast phase. In these cases as well as those detailed above, the neutrophils stained normally with FLAER, CD33, and CD14, thereby ruling out PNH, while the cells that stained with a similar intensity to normal monocytes with CD33 failed to express normal levels of CD14. Additionally these cells showed aberrant CD45 staining and bound lower levels of FLAER (data not shown).

The lower levels of FLAER staining noted on the blast populations of all these abnormal samples was very similar to levels expressed on normal CD34+ blasts detected in cytokine-mobilized peripheral blood samples. While the levels of FLAER staining in primitive normal and leukemic blast cells are higher than seen in PNH neutrophils and monocytes, such staining levels are always lower than those detected on normal neutrophils and monocytes and lymphocytes (data not shown).

DISCUSSION

Paroxysmal Nocturnal Hemoglobinuria is a rare acquired disorder of the hematopoietic stem cell (1–9). Despite its rarity, this test was performed in the UHN Clinical Flow Cytometry laboratory 141 and 164 times in the 2004 and 2005 calendar years respectively. Only three samples were unequivocally diagnosed as PNH using the Biocytex Cellquant™ and Redquant™ kits in 2004. Performance of this assay is labor intensive and costly requiring an average of more than 2 h to set up and analyze. Furthermore, the Cellquant™ assay for neutrophils

needs to be performed within 8 h of sample draw and as a large reference centre, not all our potential PNH samples could be tested within this time frame. It is not known if any PNH-positive samples were undiagnosed while this methodology was in use because of "age-of-sample" issues. In such cases, the Redquant™ assay only was performed, but the transfusion status of the patient was usually unknown at the time of testing. In cases where the Redquant™ assay generated data suggestive of PNH, a repeat sample was required to perform both parts of the kit for accurate diagnosis.

In this paper, we describe a rapid and technically simple assay utilizing FLAER in combination with CD33, CD45, and the GPI-linked structure CD14 that obviates many of the issues noted above. This assay allows the simultaneous detection of PNH clones in monocyte and granulocyte lineages. The assay takes less than 1 h from sample receipt and can be performed on a variety of bench-top instruments with four color detectors. Special software is not required for acquisition or data analysis and overall, the method is inexpensive compared to the commercial method. The assay is robust in that expression of the PNH phenotype on monocytes (FLAER-negative, CD14-negative, CD33-bright) and granulocytes (FLAER-negative, CD14-negative, CD33-weak) is stable at least up to 24 h and perhaps up to 48 h post sample draw. Similarly, non-PNH samples remain stable to analysis with this combination for 24 h and beyond and we have not detected PNH clones in any normal samples analyzed up to 48 h post sample draw. Since development and deployment of the FLAER assay in October 2004, 206 samples were tested up to December 2005 and 5 previously undiagnosed PNH cases were detected.

The assay appears to be more sensitive than the method used hitherto in our laboratory. Other previously described assays that depended on single parameter FLAER staining have claimed an assay sensitivity of about 1% (26,28). We expect that the multiparameter assay described herein will be at least as sensitive although we have not been able to formally test this on appropriate patient samples. In a number of referred samples with known PNH or other hematological diseases, we identified abnormal FLAER expression in minor populations as low as 4%.

One previously diagnosed PNH patient (PL) came in for further monitoring on two occasions and found to still have PNH monocyte and granulocyte populations in the 4–5% range. Additionally, we have been able to follow a patient (AL) with aplastic anemia over a 14-month period and were able to monitor PNH monocyte and neutrophil clones down to the 1.2–1.5% levels respectively in the most recent sample. We also identified two more patients previously diagnosed as aplastic anemia (AL and JD) with low numbers of PNH monocyte and granulocyte clones.

As expected, monocyte and granulocyte lineages showed similar proportions of PNH versus normal clones in all but one PNH sample analyzed to date. In the latter case (AS), a difference of about 20% was noted between

the more numerous monocyte population and the granulocytes. The explanation for this is unknown. In all cases in which the red cells were analyzed with the simple CD59 staining method, the proportion of PNH (type II and/or Type III) versus normal clones was always very much lower than that detected in the monocyte and/or granulocyte lineages. Potential explanations for this general observation include hemolysis of PNH red cells, particularly those with a Type III phenotype, or a history of recent red blood cell transfusion. However, in at least one case (VT) the proportion of PNH red cells (type II plus type III) was always <50% of the PNH clones detectable in either monocyte and/or granulocyte lineages even though this patient had never received a red blood cell transfusion. Thus, even in this non-transfused patient, enumeration of white blood cell lineages (granulocytes and/or monocytes) with the FLAER assay more reliably quantifies the size of the PNH clone at the hematopoietic stem cell level than the evaluation of CD55 and/or CD59 staining of red blood cells. Regardless, the CD59 assay on RBCs still has clinical utility in the monitoring of increased PNH RBCs in the context of newer therapies such as Eculizumab, a humanized monoclonal antibody that blocks the hemolysis of PNH RBCs (29).

The multiparameter FLAER-based assay described herein has also identified abnormally reduced FLAER expression in leukemic blast populations in several patient samples submitted for "PNH testing". In each case, the granulocyte population exhibited a normal staining pattern with FLAER, CD14, and CD33, quickly ruling out a diagnosis of PNH. In contrast, the CD33-bright fraction exhibited anomalies with regard to one or more of the expression of GPI-linked CD14 and/or CD45 and light scatter characteristics in addition to the unusual expression of FLAER. Reduced FLAER expression in these cases likely represents a marker of immaturity with reduced expression by leukemic blasts of GPI-linked proteins. As patients with either PNH or acute leukemia may present as pancytopenia, the flow cytometrist needs to be aware that the presence of blasts in a sample may lead to erroneous diagnosis of PNH based on FLAER expression as the sole marker for PNH detection, further supporting the requirement for a multiparameter approach. It is also recommended that whenever non-PNH hematologic abnormalities are detected with the FLAER assay, a smear of the blood sample should be made to confirm the presence of abnormal blast populations.

One important issue that needs to be addressed when developing assays for routine deployment in clinical flow laboratories is the availability of suitable material for Proficiency Testing and Quality Assurance purposes. In most jurisdictions, clinical laboratories are subjected to Laboratory Licensing through Quality Assurance and Proficiency Testing schemes. Finding standardized PNH sample material in sufficient quantities that is stable and amenable for shipping to the large number of clinical laboratories performing testing for PNH is highly problematic given the rarity of this disease. Recently however, stabilized blood samples from bona fide PNH patients have been made available by the UK NEQAS (National External Quality Assurance Scheme) organization that in preliminary testing, has been shown to be suitable for both the FLAER and CD59 red cell assays described here (unpublished data). Although preliminary, these observations suggest that if such material can be made available in sufficient quantities, a major hurdle in the standardization of PNH testing can be overcome.

In summary, we have combined FLAER with multiparameter flow cytometry to develop an improved assay for diagnosis and monitoring of PNH and detection of PNH clones in a variety of hematological diseases. This assay is sensitive, stable, obviates many of the drawbacks of other flow-based assays and represents a very simple and efficient means to detect PNH in the clinical flow laboratory.

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