Flow Cytometric Analysis of GPI-deficient Cells for the Diagnosis of Paroxysmal Nocturnal Hemoglobinuria (PNH)

Durchflußzytometrische Analyse GPI-defizienter Zellen zur Diagnose der paroxysmalen nächtlichen Hämoglobinurie (PNH)

T. Nebe[1], J. Schubert[2], K. Gutensohn[3], H. Schrezenmeier[4]

Summary: Flow cytometry is the method of choice for the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). It allows the quantitative measurement of the glycosylphosphatidyl-inositol-anchored proteins (GPI-AP) which are deficient on hematopoietic cells in this acquired stem cell disorder. It is a rapid and easy diagnostic procedure which can be applied to all hematopoietic cell lineages. However, some important pitfalls of this method have to be considered. We propose a panel of cells and GPI-AP for safe diagnosis of PNH.

Keywords: paroxysmal nocturnal hemoglobinuria; glycosylphosphatidyl-inositol anchored proteins; FACS.

Zusammenfassung: Die Durchflußzytometrie ist die Methode der Wahl für die Diagnose der paroxysmalen nächtlichen Hämoglobinurie (PNH). Diese Methode erlaubt die quantitative Messung der Glycosylphosphatidyl-Inositol verankerten Proteine (GPI-AP), welche auf den hämatopoietischen Zellen bei dieser Stammzellkrankheit fehlen. Es ist ein schnelles und einfaches diagnostisches Verfahren, welches auf alle hämatopoetischen Zellreihen angewandt werden kann. Jedoch müssen einige wichtige Problemstellen bei dieser Methode beachtet werden. Wir schlagen ein Spektrum von Zellen und GPI-AP für die sichere Diagnose der PNH vor.

Schlüsselwörter: paroxysmale nächtliche Hämoglobinurie; Glycosylphosphatidyl-inositol verankerte Proteine; FACS.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder of hemopoiesis [1]. The genetic basis of PNH are acquired somatic mutations in the PIG-A gene of primitive hematopoietic progenitor cells [2–5]. The gene product of the PIG-A gene is essential for the biosynthesis of the glycosylphosphatidyl-inositol anchor (GPI) in the endoplasmatic reticulum [6]. Proteins that are linked to the cell membrane by GPI anchors are lacking on the cell surface of PNH cells [7]. In the bone marrow, there is a mosaic of PIG-A-mutated and normal stem cells which leads to a coexistence of GPI-deficient and normal blood cells [8].

In all PNH patients analyzed so far, a PIG-A gene mutation could be demonstrated as the basis of the GPI-AP deficient phenotype. A great variety of mutations has been reported (point mutations, deletions, insertions) [9]. Small deletions and insertions leading to a frameshift prevail. PIG-A gene mutations are scattered over the whole coding region of the PIG-A gene. Apart from a five-base pair deletion in exon 2 which was reported in aplastic anemia patients with a GPI-deficient population, there is no mutational hot spot [10]. In some patients several PNH clones with different mutations coexist [11, 12].

The clinical course of PNH is highly variable. It is characterized by chronic or acute hemolysis, thrombosis and bone marrow failure with neutropenia and thrombocytopenia (all combinations of these symptoms can occur) [13–15].

Hemolysis can be explained by the deficiency of complement-regulating proteins on red cells (CD55, CD59) which are GPI-anchored. Since erythrocytes do not express MCP (membrane cofactor protein, CD46-molecule), they are abnormally sensitive to the activitation of the autologous complement cascade leading to hemolysis [16].

The reason for the increased incidence of venous thromboses is not fully understood. Cytoplasmic components released from erythrocytes are known to be prothrombogenic. Upon complement activation there is an increased release of microvesicules from platelets and exposure of membrane prothrombinase sites [17, 18]. Varying degrees of platelet activation as defined by the surface expression of activation-dependent proteins and the binding of adhesive proteins to the platelet surface are present in PNH patients [19]. Absence of the GPI-anchored protein urokinase-like plasminogen acti-

[1]Abt. Klinische Chemie, Universitätsklinikum Mannheim; [2]Abt. Immunologie, Medizinische Hochschule Hannover; [3]Gemeinschaftspraxis Prof. Heike und Kollegen, Ingelheim; [4]Abt. Transfusionsmedizin, Universitätsklinikum Ulm
Correspondence: Dr. med. Carl Thomas Nebe, Institut für Klinische Chemie, Theodor-Kutzer -Ufer 1–3, 68167 Mannheim, Germany
Fax: +49 62 13 83-38 19
E-mail: thomas.nebe@ikc.ma.uni-heidelberg.de

vator (uPAR, CD86) on PNH monocytes might lead to reduced fibrinolytic activity [16].

Increased intravascular hemolysis leads to hemoglobinuria and hemosiderinuria which ultimately results in iron deficiency in most of the patients.

A striking feature of PNH is its clinical association with aplastic anemia and myelodysplasia [14, 20, 21]. Up to 65 % of patients with primary diagnosis of PNH present with bicytopenia or pancytopenia and a substantial proportion will develop an aplastic bone marrow during the course of disease [14, 21]. Vice versa, a significant GPI-deficient cell population can be demonstrated in up to 50 % of patients with aplastic anemia [22–27] and about 15 % of aplastic anemia patients who are long-term survivors after immunosuppressive therapy develop clinical PNH [28, 29]. The relative proportion of GPI-deficient cells is lower in patients with aplastic anemia and a coexistent PNH clone as compared to patients with primary clinical PNH [23, 27].

Diagnosis of PNH can be suspected in all cases of unexplained acquired hemolysis with a negative Coombs test, in cases of atypical thrombosis (Budd-Chiari-Syndrome, mesenterial thrombosis, sinus thrombosis) or thrombosis associated with hemolysis, in patients with unexplained iron deficiency associated with hemolysis and in patients with hemolysis associated with bone marrow failure.

Hemolysis tests based on the increased susceptibility to complement-mediated lysis (acidified serum test; "Ham's test", sucrose lysis test) have been the gold standard of PNH diagnosis [30]. Since elucidation of the molecular basis of PNH, hemolysis tests have been replaced in most labs by flow cytometric analysis of GPI-anchored proteins on blood cells.

Flow cytometric measurement of GPI-positive and -negative cells provides several advantages over the classical hemolysis tests: Whereas hemolysis tests are confined to detection of the defect on red cells, flow cytometry allows separate analysis of all lineages (including progenitor cells which might be important in treatment approaches employing stem cell transplantation) [24, 31–36]. In most cases the proportion of affected granulocytes is higher than the proportion of affected erythrocytes [37, 38] and the proportion of affected reticulocytes is higher than the proportion of erythrocytes [36, 39, 40]. Detection of the PNH clone in erythrocytes (both by hemolysis tests and flow cytometry) might be impaired by red blood cell transfusions or acute hemolytic events. This can lead to false-negative results or underestimation of the size of the PNH clone. Flow cytometry allows separate analysis of expression of GPI-anchored proteins on reticulocytes [36]. The proportion of GPI-deficient cells (i. e. the size of the PNH clone) can easily be quantitated in all affected lineages. This can be important to monitor effects of treatment on the PNH clone.

Principles of Flow Cytometric Analysis

Selection of cells

The deficiency of GPI-anchored cell surface proteins (GPI-AP) may be found on all lineages of blood cells with a mosaic of normal and deficient cells [24, 31–36]. The proportion of the two populations can be different in the various lineages. ETDA-anticoagulated peripheral blood is the sample of choice.

Erythrocytes and reticulocytes are of major interest. The analysis of GPI-deficient reticulocytes results in a higher sensitivity and avoids false-negative results or underestimation of the size of the PNH clone in transfused patients or patients during/after an acute hemolytic crisis [36, 39–41].

Among the leukocytes, the neutrophils are the most relevant population because they represent more closely the proportion of normal and GPI-deficient bone marrow progenitor cells [37, 38]. The proportion of deficient lymphocytes is much lower compared to other lineages. Monocytes are more difficult to analyze because they are heterogeneous and hard to define purely by means of light scatter characteristics. In addition, analysis of monocytes does not provide additional information to the analysis of neutrophils.

Autologous transplantation of unaffected progenitor cells after high-dose chemotherapy (e. g. high-dose cyclophosphamide) might become a new therapeutic option for treatment of PNH. However, this approach would require selection of unaffected autologous progenitor cells. Therefore, the analysis of expression of GPI-anchored proteins on CD34 positive cells from bone marrow or peripheral blood after mobilization and from leukapheresis products has to be considered [42, 43].

For flow cytometric analysis, the major cell populations in peripheral blood are commonly defined by forward and orthogonal light scatter characteristics. However, for detection of small subpopulations (i. e. in aplastic anemia) impurities of scatter gates have to be considered and additional markers to be added.

For screening purposes, it is recommended to analyze GPI-AP on erythrocytes and neutrophils. In case of a significant GPI-AP-deficient population in these lineages a more detailed analysis including reticulocytes and platelets should be performed for future monitoring of the size of the affected population.

Definition of cellular subsets

For normal routine analysis, blood cells are commonly defined and gated by software based solely on their light scatter characteristics. However, there are impurities like reagent aggregates and debris in these scatter cell populations which might be misinterpretated as deficient cells, especially in situations like aplastic anemia when small fractions of affected cells may be searched. Therefore, gating strategies including immunological parameters should be preferred. CD41 or CD61 may be used for platelets, glycophorin A for red cells, RNA

dyes for reticulocytes, CD3 or CD45 for lymphocytes and CD33 for monocytes (bright) and neutrophils (dim). These parameters are displayed versus side scatter and possibly forward scatter as a third parameter to define and gate these cells for further analysis of GPI-AP.

Selection of GPI-anchored antigens
There is a growing list of monoclonal antibodies clustered by the leukocyte antigen workshops that define GPI-anchored molecules (see Table 1). The protein recognized by the antibody should fulfil several criteria to allow a safe diagnosis of PNH:
– expression on all subpopulations of the respective lineage of blood cells
– sufficient high expression (cell surface density)
– expression on all cells independent of the maturational stage
– absence of a genetic polymorphism
– no posttranscritional processing (e. g. CD16, FcγRIII)
The antigens which best meet these requirements and are therefore preferred are CD58 and CD59 for analysis of red cells, CD55 for platelets CD55, and CD24 and CD66b for neutrophils.

Selection of Antibodies and Conjugates
The antibody clones used should have been clustered by one of the leukocyte differentiation antigen workshops or extensively tested by a laboratory experienced in the flow cytometric analysis of PNH.

Optimal results will be obtained if monoclonal antibodies recognizing more dimly expressed antigens like CD55 are conjugated with bright fluorescent fluorochromes i. e. the phycobiliprotein R-Phycoerythrin (PE) or its tandem conjugates like PE-Cyanine5. Directly conjugated antibodies are preferable to indirect labeling techniques in order to avoid agglutination, in particular of red cells.

The above-mentioned considerations lead to the following list of reagents (Table 2).

Table 1 Candidate cell types and markers analysed for flow cytometric diagnosis of PNH

Cell Type	Definition	1st Choice	2nd Choice	3rd Choice
Red Cells				
Reticulocytes	TO/light scatter	CD59	CD58	CD55
Erythrocytes	GpA/light scatter	CD59	CD58	CD55
Leukocytes				
Neutrophils	light scatter/ CD33	CD66b	CD24	CD16
Monocytes	light scatter/ CD33	CD14	CD48	
Lymphocytes	light scatter/ CD3	CD48	CD52	
Progenitor Cells	CD34/CD45	CD59		
Platelets	LS/CD41, 42 or 61	CD55-PE		

Controls
The analysis of a healthy donor and the use of isotype controls show that the test has been done properly. The monitoring of antigen density, i. e. mean fluorescence intensity over time indicates the deterioration of the antibody in use. This is important because the test may be done infrequently and in particular the PE conjugates are prone to bacterial degradation and the PE-Cy5 tandem conjugates are light sensitive.

As mentioned above, the use of a second monoclonal antibody against GPI-anchored proteins and analysis of separate blood cell lineages is an important aspect. In addition other laboratory data of intravasal hemolysis provide further plausibility (see Table 3).

Current problems in the diagnosis of GPI deficiency
Detection of a low frequency of GPI-deficient cells is a challenge. This can occur early in disease (esp. when evolving from aplastic anemia), after "dilution" of GPI-deficient cells by transfusion or in monitoring of minimal residual disease after allogeneic stem cell transplantation. Therefore careful determination of cut-off values for each blood cell population and antibody conjugate is necessary. The respective mean number of negative cells in the analysis gate in healthy controls plus two standard deviations is a safe limit.

The effect of various different commercial lysing reagents has not been tested yet. As known from other applications (e. g. like progenitor cell counting) the antigen may be modified in a way that results in reduced binding of the test antibody.

Protocol for the Flow Cytometric Analysis

Required information
The basic clinical information required is the information of the current clinical diagnosis, preceding transfusions or transplantation. It should be stated that the sample is derived from peripheral blood and the time of blood drawing should be noted to avoid the analysis of aged blood.

Preanalytical phase
The anticoagulant should be EDTA and the sample stored at stabilized room temperature. 4–10 °C is preferrable for overnight storage. The test may be performed within 24 hours but the complete blood count should be done within the first 6 hours. For reasons of sensitivity the test should be done before transfusions are given.

Table 2 GPI-anchored membrane proteins

CD	Designated	Distribution	Function/Remarks
CD14		monocytes neutrophils	receptor for LPS-binding protein expr. Maturation and activation-dependent
CD16	FcRIII	neutrophils monocytes	low affinity receptor for IgG expr. Maturation and activation-dependent
CD16		NK cells	on NK cells transmembraneously-anchored
CD24		neutrophils B cells	maturation dependent unknown function
CD48	Blast1	monocytes lymphocytes	adhesion, stabilizes TCR complex
CD52	Campath-1	lymphocytes	partially on granulocytes, eosinophils
CD55	DAF	erythrocytes leukocytes	decay accelerating factor for complement
CD58	LFA3	erythrocytes leukocytes	expressed in many cells in the body CD2 ligand, target-killer adhesion
CD59	MIRL	erythrocytes leukocytes	membrane inhibitor of reactive lysis
CD66b	(CD67)	all granulocytes	activation of neutrophils
CD73		lymphocytes	ecto-5′-nucleotidase, membrane bound enzyme
C8bp		erythrocytes	complement factor C8 binding protein
Acetylcholinesterase		erythrocytes	membrane-bound enzyme
Alkal. Phosphatase		neutrophils	membrane-bound enzyme
CD90 (Thy1)		stem cells	adhesion?
CD108		erythrocytes lymphcytes	(JMH blood-group antigen) adhesion molecule
CD109		T lymphocytes platelets progenitor cells	

Sample preparation
Whole blood is used for all tests (red cells, leukocytes or platelets) requiring subsequent lysis only when leukocytes are analysed.

Erythrocytes
In case of the analysis of red cells 10 µl of 1:10 diluted whole blood are used for the incubation for 15 minutes at room temperature. Saturating amounts have to be checked by pre-titration of antibodies. The incubation is followed by one washing step with phosphate buffered saline (PBS). The pellet is diluted with 0.5 ml of PBS for the analysis of erythrocytes or incubated for 15 minutes with 0.5 ml of thiazol orange (0.1 µg/ml) or reticount™ solution (Becton Dickinson) when reticulocytes are investigated. The samples should be analyzed within two hours.

Thrombocytes
The test is set up in a similar manner combining a platelet marker (eg. CD61-FITC) with a GPI protein (CD55-PE).

Leukocytes
50 µl of EDTA blood (100 µl for progenitor cells) are incubated with 20 µl of the respective antibody at saturating amounts. After 15 minutes of incubation at room temperature in the dark lysing solution is added (2 ml FACS Lysing solution™ Becton Dickinson) and incubated for further 10 minutes followed by a washing step with PBS.

Measurement

The statistical precision wanted determines the number of events that need to be collected. Commonly two different instrument settings are necessary to analyze platelets and red cells on one hand and leukocytes on the other. For erythrocytes and thrombocytes, all parameters are displayed in a logarithmic fashion and in dot plot mode. For gating leukocyte populations depending on the instrument it may be advantageous to use linear scales of scatter parameters for a better cluster resolution (see Fig. 1–4).

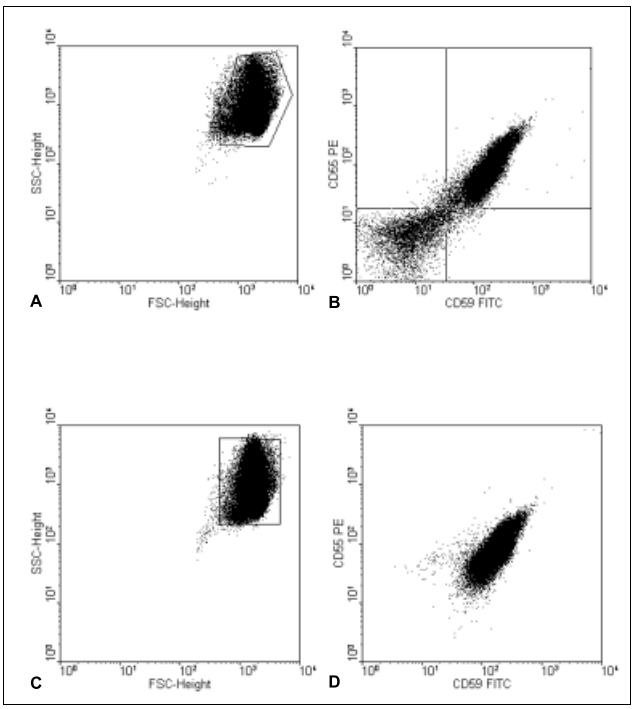

Figure 1 Typical test results of red cells from a patient with GPI-AP defciency (A, B) and a normal donor (C, D).

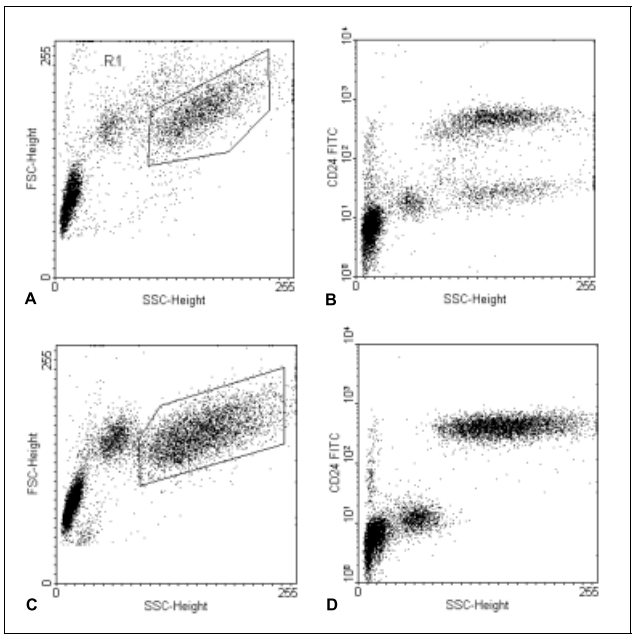

Figure 3 Typical test results of neutrophils from the same patient (GPI-AP defciency A, B) and a normal donor (C, D).

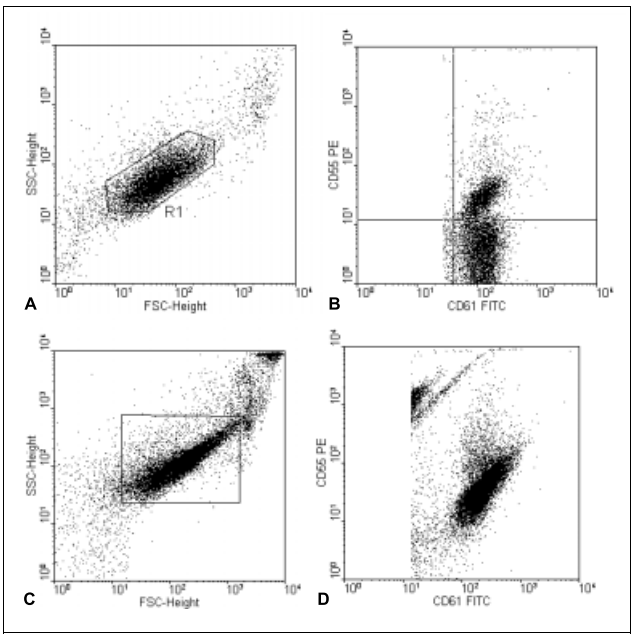

Figure 2 Typical test results of platelets from a patient with GPI-AP defciency (A, B) and a normal donor (C, D).

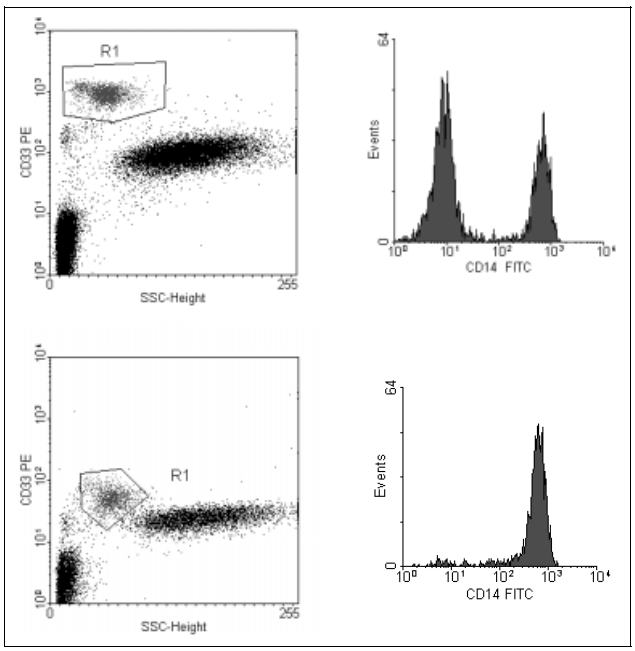

Figure 4 shows typical test results of monocytes from the same patient (GPI-AP defciency 1A, 1B) and a normal donor (1C, 1D). The data set of the normal donor illustrates the problem of gating monocytes by independent means (CD33). A small portion is CD14 negative.

The amplification is set up in a way that the negative population in terms of fluorescence is completely visible (i. e. in the first decade of the four log scale). Because the unstained cells are diagnostic, it is helpful to run a test tube containig PBS only to localize the noise of the instrument or debris particles.

Fluorescence compensation may be done either by using calibration beads or cell mixtures stained with mutual exclusive antibodies (eg. CD4 and CD8 on lymphocytes). Further appropriate measures should be taken to ensure internal quality control. Participation in schemes for external quality assessment (EQA) is recommended, however, specific EQAs for GPI antigens do not yet exist.

Data evaluation

A software analysis region with gating function is set around the population of interest and the fluorescence histogram of the respective GPI-anchored antigen is displayed. The negative population is defined by staining with an appropriate isotype control directed against an irrelevant antigen. The positive fraction is verified by the cells of a healthy donor labeled with an antibody directed against a GPI-AP.

Reporting of results

It is advantageous to report the result of GPI-anchored proteins in the context of other laboratory results that prove intravasal hemolysis (see Table 3). Therefore the document should include at least the hematology results:

Complete blood count: Hb, RBC, MCV, platelets
reticulocytes: percentage and absolute counts
GPI-anchored antigens: percentage of deficient cells
– on reticulocytes
– on erythrocytes
– on neutrophils
– on platelets

naming the CD number, the name of the clone and the type of conjugate (FITC, PE, APC or Tandem).

A review of the blood film may give valuable information indicating other sources of anemia when GPI-AP are expressed normally.

Interference

Contamination of the analysis gate with unwanted cells is a major source of error. For example, the red cell scatter gate is contaminated with white cells and macrothrombocytes, the neutrophil gate may include eosino-

Table 3 Laboratory parameters in the diagnosis of PNH

Parameter	Material	Remarks
Increased		
Reticulocytes	EDTA blood	in primary PNH (> 5 %), may be low in AA
Bilirubin	serum	especially indirect bilirubin
LDH	serum	especially during hemolytic crisis
Free Hb	serum	
Urobilinogen	urine	together > 3 mg/day
Urobilin	urine	
Stercobilinogen	stool	together > 280 mg/day
Stercobilin	stool	
Decreased		
Erythrocytes	EDTA blood	
Leukocytes	EDTA blood	neutrophils
Thrombocytes	EDTA blood	variable
Hemoglobin	EDTA blood	often 7–8 g/dl
Haptoglobin	serum	
Normal		
Coombs Test	whole blood	direct test negative (no autoantibodies)
Cold Antibodies	whole blood	season!
Osmot. resistance	EDTA blood	normal fragility

phils or immature myeloid progenitors which do not express CD16 or CD24. The lymphocyte gate may contain unlysed red cells, normoblasts, macrothrombocytes and/or basophils and the monocyte gate is hard to define by light scatter anyhow. Analysis of platelets require particle-free reagents i. e. antibodies, washing solutions or sheath fluid. To avoid these problems, a combination with an immunological marker that define the cell population in question is recommended. CD4 or CD33 for monocytes and esp. CD41 (or CD42 or CD61). If deemed necessary (because of pathological differential or counts), for red cells Glycophorin A, for neutrophils CD15 and for lymphocytes CD3 can be used.

Heparinized blood often induces severe platelet aggregation that interferes with most analysis gates and is therefore not recommended.

Discussion

Immunophenotypic analysis of the loss of GPI-anchored proteins by flow cytometry is the method of choice for the diagnosis of the life threatening disease PNH. If the recommendations and pitfalls mentioned here are considered the method is safe.

Flow cytometry of GPI-anchored proteins should be performed in all cases of otherwise unexplained Coombs-negative acquired hemolytic anemia, occurrence of atypical thromboses (in particular Budd-Chiari Syndrome, portal vein and mesenterial thrombosis, sinus thrombosis) in the absence of other risk factors for these conditions, occurrence of hemolysis in association with thrombosis, cytopenia or unexplained iron deficiency. PNH takes a variable course in terms of symptoms and progression [13–15]. Even spontaneous remissions can occur [13, 25]. Therefore, a continuous monitoring of the patients is required. The time interval may be 6 months in case of an uneventful course. However, if there is deterioration of clinical symptoms (e. g. hematolytic crisis, thrombotic events) or if monitoring after specific treatment is required (e. g. cytokine treatment, immunosuppression or stem cell tranplantation), flow cytometry might be repeated after shorter intervals.

As the PNH syndrome may develop at any time during the course of aplastic anemia regular testing (at initial presentation and thereafter at about 6-monthly intervals) is recommended also in this disease [25].

To limit the cost and time efforts we suggest a test sequence: A screening should be done on red cells and neutrophils with at least two GPI-anchored markers on each lineage. In case of a positive result in one or both lineages a detailed analysis of reticulocytes and perhaps platelets should follow. Analysis of monocytes is optional since there is no evidence that analysis of monocytes is more informative than analysis of neutrophils. Because the number of affected lymphocytes is low (if involved at all), we do not recommend their analysis for routine testing.

Problems arise in the analysis of neutrophils in case of a pathological left shift (e. g. myelodysplastic syndromes, myeloproliferative syndromes) since immature myeloid cells in the granulocyte gate might be misinterpreted as GPI-deficient cells (e. g. CD16 negative cells) while the absence of a particular GPI-anchored proteins is not due to a real GPI deficiency but due to the maturational stage of the cells. Therefore a second marker should be employed which is also expressed on myeloid progenitor cells (CD66b). Similarly, it must be avoided to falsely interpret eosinophils which are physiologically CD16-negative as GPI-deficient cells. It can also become difficult to identify a small proportion of deficient cells in the beginning of PNH syndrome in aplastic anemia [24, 25]. This requires careful establishment of cut-off values for GPI-deficient populations in the various lineages.

PNH diagnosis has to be confirmed by assessment of at least two different GPI-anchored proteins on two lineages. Results with the different markers on one lineage must be concordant, i. e. the respective population must show reduced expression of all GPI-anchored proteins tested. If there is a deficiency of only one GPI-anchored protein this should not be diagnosed as PNH. If there are discordant results (i. e. not all GPI-anchored proteins show reduced or absent expression), in most cases this is due to contamination of the analyzed region by other cells (e.g. myeloid progenitors or eosinophils in the neutrophil region; nucleated red cells in the lymphocyte region etc.). Discordant results can also be due to a defect in the respective protein (and not a defect of the GPI anchor), as for expample an isolated CD59 defect [44].

Polymorphism is known for CD16 but other rarely tested GPI-AP may also show this feature and therefore caution needs to be taken when different clones of antibodies of the same CD are used.

Neutrophils were affected in all cases of clinical PNH reported so far and in the vast majority of cases the largest proportion of affected cells is present in neutrophils [24, 36–38]. Therefore, rare cases of GPI deficiency in other populations without affected neutrophils should be subjected to further analysis (including PIG-A-gene) in specialized laboratories.

Sometimes, there is no significant erythrocyte population with GPI deficiency in a patient with a clear GPI deficiency on neutrophils. This might happen in particular after severe hemolytic crises and transfusion of packed red cells. However, in this situation analysis of reticulocytes demonstrate a GPI defect in most of the cases. Overall, there is a better correlation between the proportion of affected neutrophils and reticulocytes as compared to neutrophils and erythrocytes [24, 36–38].

In the past, hemoysis tests (acidified serum test, sucrose lysis test) have been the gold standard for laboratory diagnosis of PNH. There are several advantages of flow cytometry over hemolysis tests. Flow cytometry directly leads to the detection of the molecular basis of the disease. Hemolysis tests are confined to red cells

whereas flow cytometry allows analysis of all lineages. This is particularly important when analysis of erythrocytes is hampered by hemolytic crisis or transfusions. Flow cytometry easily allows exact assessment of the size of the PNH clone and monitoring of the proportion of GPI-deficient cells during the course of the disease, which is in particular relevant when assessing the effect of therapeutic interventions on the PNH clone.

It has been demonstrated that many patients with acquired bone marrow failure syndrome and myelodysplastic syndrome harbor a significant GPI-deficient population, however, many of these patients do not have clinical PNH with hemolysis or thrombosis [22–27]. It has been demonstrated that very small numbers of GPI-deficient cells might even be present in healthy persons [45]. The increasing use of flow cytometric tests of GPI-anchored proteins leads to detection of a substantial number of patients with a significant proportion of GPI-deficient cells but without clinical PNH symptoms. This situation should be termed as "flow cytometric PNH" (in contrast to clinical PNH with typical PNH disease activity and symptoms). Information on course, complications and survival of the disease that is available in the literature refers to "clinical PNH" only [46]. Prognostic implications of a PNH purely based on laboratory findings are less clear and need to be studied in prospective investigations with long follow-up.

The analysis of bone marrow for diagnosis of PNH is not established and therefore not yet recommended. Future developments should include the analysis of bone marrow, hemopoietic stem cells and leukocytes after apheresis and immunoselection procedures.

The number of laboratories performing flow cytometric analysis of GPI-anchored proteins is increasing. The guidelines described here should help to provide reproducible and comparable results among different laboratories. They aim at improvement and standardization of routine assessments for diagnosis and monitoring of PNH. Research protocols (e. g. new approaches for treatment, basis research on pathophysiology) might require more sophisticated antibody panels or isolation procedures.

New developments aim at direct detection of the deficiency of the GPI anchor itself rather than detection of deficiency of GPI-anchored proteins. Some bacterial toxins which bind to the glycan core of GPI anchors with high specificity and high affinity might be used as tools to measure expression of GPI anchors. One of these toxins is aerolysin from *Aeromonas hydrophilia*. It allows sensitive detection of GPI-deficient populations [47, 48] and there is a good correlation of results obtained by methods detecting GPI-anchored proteins and methods using a flourescent variant of aerolysin [49]. Thus it is a promising candidate for the use in routine flow cytometric diagnosis of PNH.

References

1. Hillmen P, Richards SJ. Implications of recent insights into the pathophysiology of paroxysmal nocturnal haemoglobinuria. Br J Haematol 2000;108:470–9.

2. Bessler M, Mason PJ, Hillmen P, Miyata T, Yamada N, Takeda J, Luzzatto L, Kinoshita T. Paroxysmal nocturnal haemoglobinuria (PNH) is caused by somatic mutations in the PIG-A gene. EMBO J 1994;13:110–7.

3. Kinoshita T, Inoue N, Takeda J. Role of phosphatidylinositol-linked proteins in paroxysmal nocturnal hemoglobinuria pathogenesis. Annu Rev Med 1996;47:1–10.

4. Miyata T, Yamada N, Iida Y, Nishimura J, Takeda J, Kitani T, Kinoshita T. Abnormalities of PIG-A transcripts in granulocytes from patients with paroxysmal nocturnal hemoglobinuria [see comments]. N Engl j Med 1994;330:249–55.

5. Späth-Schwalbe E, Schrezenmeier H, Heimpel H. Paroxysmale nächtliche Hämoglobinurie: Klinische Erfahrungen bei 40 Patienten in einem Zentrum über 25 Jahre. Dtsch.Med.Wochenschr 1995;120: 1027–33.

6. Ware RE, Rosse WF, Hall SE. Immunophenotypic analysis of reticulocytes in paroxysmal nocturnal hemoglobinuria. Blood 1995; 86:1586–9.

7. Rosse WF. Paroxysmal nocturnal hemoglobinuria as a molecular disease. Medicine Baltimore. 1997;6:63–93.

8. Luzzatto L, Bessler M. The dual pathogenesis of paroxysmal nocturnal hemoglobinuria. Curr Opin Hematol 1996;3:101–10.

9. Nafa K, Bessler M, Castro-Malaspina H, Jhanwar S, Luzzatto L. The spectrum somatic mutations in the PIG-A gene in paroxysmal nocturnal hemoglobinuria includes large deletions and small duplications. Blood Cells Mol Dis 1998;24:370–84.

10. Mortazavi Y, Merk B, McIntosh J, Marsh JCW, Schrezenmeier H. The spectrum of PIG-A gene mutations in aplastic anemia/paroxysmal nocturnal haemoglobinuria (AA/PNH). A high incidence of multiple mutations and evidence of a mutational hotspot. Blood 2003;101:2833–41.

11. Endo M, Ware RE, Vreeke TM, Singh SP, Howard TA, Tomita A, Holguin MH, Parker CJ. Molecular basis of the heterogeneity of expression of glycosyl phosphatidylinositol anchored proteins in paroxysmal nocturnal hemoglobinuria. Blood 1996;87:2546–57.

12. Nebe CT. Paroxysmal Nocturnal Hemoglobinuria: Clinical Aspects and Flow Cytometric Analysis. In: Aspects of the Flow-Cytometric Analysis of Red Blood Cells, K. Gutensohn, H.-H. Sonneborn and P. Kühnl (eds.), Clin. Lab. Publications 1997:81–94.

13. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1995;333:1253–8.

14. Socie G, Henry Amar M, Bacigalupo A, Hows J, Tichelli A, Ljungman P, McCann SR, Frickhofen N, Van't Veer Korthof E, Gluckman E. Malignant tumors occurring after treatment of aplastic anemia. European Bone Marrow Transplantation-Severe Aplastic Anaemia Working Party. N Engl J Med 1993;329:1152–7.

15. Socie G, Mary JY, De Gramont A, Rio B, Leporrier M, Rose C, Heudier P, Rochant H, Cahn JY, Gluckman E. Paroxysmal nocturnal haemoglobinuria: long-term follow-up and prognostic factors. French Society of Haematology [see comments]. Lancet 1996;348: 573–7.

16. Rosse WF. Dr Ham's test revisited. Blood 1991;78:547–50.

17. Hugel B, Socie G, Vu T, Toti F, Gluckman E, Freyssinet JM, Scrobohaci ML. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. Blood 1999;93:3451–6.

18. Watanabe R, Kinoshita T, Masaki R, Yamamoto A, Takeda J, Inoue N. PIG-A and PIG-H, which participate in glycosylphosphatidylinositol anchor biosynthesis, form a protein complex in the endoplasmic reticulum. J Biol Chem 1996;271:26 868–75.

19. Gralnick HR, Vail M, McKeown LP, Merryman P, Wilson O, Chu I, Kimball J. Activated platelets in paroxysmal nocturnal haemoglobinuria. Br J Haematol 1995;91:697–720.
20. Harris JW, Koscick R, Lazarus HM, Eshleman JR, Medof ME. Leukemia arising out of paroxysmal nocturnal hemoglobinuria. Leuk Lymphoma 1999;32:401–26.
21. Prince GM, Nguyen M, Lazarus HM, Brodsky RA, Terstappen LW, Medof ME. Peripheral blood harvest of unaffected CD34+ CD38- hematopoietic precursors in paroxysmal nocturnal hemoglobinuria. Blood 1995;86:3381–6.
22. Dunn DE, Tanawattanacharoen P, Boccuni P, Nagakura S, Green SW, Kirby MR Kumar MS, Rosenfeld S, Young NS. Paroxysmal nocturnal hemoglobinuria cells in patients with bone marrow failure syndromes [see comments]. Ann Intern Med 1999;131:401–8.
23. Griscelli Bennaceur A, Gluckman E, Scrobohaci ML, Jonveaux P, Vu T, Bazarbachi A, Carosella ED, Sigaux F, Socie G. Aplastic anemia and paroxysmal nocturnal hemoglobinuria: search for a pathogenetic link. Blood 1995;85:1354–63.
24. Rosse WF, Ware RE. The molecular basis of paroxysmal nocturnal hemoglobinuria. Blood 1995;86:3277–86.
25. Schrezenmeier H, Hertenstein B, Wagner B, Raghavachar A, Heimpel H. A pathogenetic link between aplastic anemia and paroxysmal nocturnal hemoglobinuria is suggested by a high frequency of aplastic anemia patients with a deficiency of phosphatidylinositol glycan anchored proteins. Exp Hematol 1995;23:81–7.
26. Schubert J, Scholz C, Geissler RG, Ganser A, Schmidt RE. G-CSF and cyclosporin induce an increase of normal cells in hypoplastic paroxysmal nocturnal hemoglobinuria. Ann Hematol 1997; 74:225–30.
27. Wiedmer T, Hall SE, Ortel TL, Kane WH, Rosse WF, Sims PJ. Complement-induced vesiculation and exposure of membrane prothrombinase sites in platelets of paroxysmal nocturnal hemoglobinuria. Blood 1993;82:1192–6.
28. Schubert J, Vogt HG, Zielinska Skowronek M, Freund M, Kaltwasser JP, Hoelzer D, Schmidt RE. Development of the glycosylphosphatitylinositol-anchoring defect characteristic for paroxysmal nocturnal hemoglobinuria in patients with aplastic anemia. Blood 1994;83:2323–8.
29. Takeda J, Miyata T, Kawagoe K, Iida Y, Endo Y, Fujita T, Takahashi M, Kitani T, Kinoshita T. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell 1993;73:703–11.
30. Raghavachar A, Schrezenmeier H. The aplastic anemia – paroxysmal nocturnal hemoglobinuria syndrome. In: Gluckman E, Coulombel L (eds), Ontogeny of hematopoiesis – Aplastic anemia, London, Paris, John Libbey Eurotext, 1995: 233.
31. Navenot JM, Bernard D, Harousseau JL, Muller JY, Blanchard D. Expression of glycosyl-phosphatidylinositol-linked glycoproteins in blood cells from paroxysmal nocturnal haemoglobinuria patients: a flow cytometry study using CD55, CD58 and CD59 monoclonal antibodies. Leuk Lymphoma 1996;21:143–51.
32. Piedras J, Lopez-Karpovitch X. Flow cytometric analysis of glycosylphosphatidyl-inositol-anchored proteins to assess paroxysmal nocturnal hemoglobinuria clone size. Cytometry 2000;42:234–8.
33. Schrezenmeier H, Koerper S, Punnose A, Krause K, Rojewski M, Thiel E. Fluorescently labeled variant of aerolysin (FLAER): A new method for detection of GPI-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and aplastic anemia (AA). Onkol 2001 (abstr.).
34. Schubert J, Alvarado M, Uciechowski P, Zielinska Skowronek M, Freund M, Vogt H, Schmidt RE, Diagnosis of paroxysmal nocturnal haemoglobinuria using immunophenotyping of peripheral blood cells. Br J Haematol 1991;79:487–92.
35. Tichelli A, Gratwohl A, Nissen C, Speck B. Late clonal complications in severe aplastic anemia. Leuk Lymphoma 1994;12:167–75.
36. van der Schoot CE, Huizinga TW, van't Veer Korthof ET, Wijmans R, Pinkster J, von dem Borne AE. Deficiency of glycosylphosphatidylinositol-linked membrane glycoproteins of leukocytes in paroxysmal nocturnal hemoglobinuria, description of a new diagnostic cytofluorometric assay. Blood 1990;76:1853–9.
37. Nishimura J, Inoue N, Wada H, Ueda E, Pramoonjago P, Hirota T, Machii T, Kageyama T, Kanamaru A, Takeda J, Kinoshita T, Kitani T. A patient with paroxysmal nocturnal hemoglobinuria bearing four independent PIG-A mutant clones. Blood 1997;89:3470–6.
38. Pakdeesuwan K, Wanachiwanawin W, Siripanyaphinyo U, Pattanapanyasat K, Wilairat P, Issaragrisil S. Immunophenotypic discrepancies between granulocytic and erythroid lineages in peripheral blood of patients with paroxysmal nocturnal haemoglobinuria. Eur J Haematol 2000;65:8–16.
39. Iwamoto N, Kawaguchi T, Nagakura S, Takatsuki K, Nakakuma H. Markedly high population of affected reticulocytes negative for decay accelerating factor (DAF) and CD59 in paroxysmal nocturnal hemoglobinuria. Blood 1995; 84(Suppl.1):11a, (abstr.).
40. Schrezenmeier H, Hildebrand A, Rojewski M, Hacker H, Heimpel H, Raghavachar A. Paroxysmal nocturnal haemoglobinuria: a replacement of haematopoietic tissue? Acta Haematol 2000;103:41–8.
41. Navenot JM, Muller JY, Blanchard D. Investigation of the survival of paroxysmal nocturnal hemoglobinuria red cells through the immunophenotyping of reticulocytes. Transfusion 1998;38:337–42.
42. Hillmen P, Richards SJ. Implications of recent insights into the pathophysiology of paroxysmal nocturnal haemoglobinuria. Br J Haematol 2000;108(3):470–9.
43. Schubert J, Schmidt RE. [Paroxysmal nocturnal hemoglobinuria. Diagnosis by fluorescence-activated cell analysis]. Dtsch Med Wochenschr 1992;117:985–9.
44. Yamaguchi M, Machii T, Azenishi Y, Nishimura J, Shibano M, Kanakura Y, Kitani T. Detection of small populations of CD59-deficient erythrocytes in patients with aplastic anemia or myelodysplastic syndrome and normal individuals. Blood Cells Mol Dis 2000;26:247–54.
45. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. Proc Natl Acad Sci USA 1999;96:5209–14.
46. Plesner T, Hansen NE, Carlsen K. Estimation of PI-bound proteins on blood cells from PNH patients by quantitative flow cytometry. Br J Haematol 1990;75:585–90.
47. Brodsky RA, Mukhina GL, Li S, Nelson KL, Chiurazzi PL, Buckley JT, Borowitz MJ. Improved detection and characterization of paroxysmal nocturnal hemoglobinuria using fluorescent aerolysin. Am J Clin Pathol 2000;114:459–66.
48. Brodsky RA, Mukhina GL, Nelson KL, Lawrence TS, Jones RJ, Buckley JT. Resistance of paroxysmal nocturnal hemoglobinuria cells to the glycosylphosphatidylinositol-binding toxin aerolysin. Blood 1999;93:1749–56.
49. Schrezenmeier H, Iliewa S, Prümmer O, Raghavachar A, Heimpel H. Deficiency of glycosylphosphatidyl-inositol anchored proteins (GPI-AP) in aplastic anemia (AA): High frequency of GPI-AP deficient reticulocytes and higher sensitivity of flow cytometric analysis of reticulocytes compared to hemolysis tests for detection of a PNH clone in AA. Blood 1995;86:131a (abstr.).