Flow cytometric analysis of CD55 and CD59 expression on blood cells in paroxysmal nocturnal haemoglobinuria

Grzegorz Dworacki¹, Jan Sikora¹, Ewa Mizera-Nyczak¹, Magdalena Trybus¹, Iwona Mozer-Lisewska², Anna Czyz³ and Jan Zeromski¹

Departments of ¹Clinical Immunology, ²Child Infectious Diseases and ³Haematological Haemopoietic Proliferative Diseases, University of Medical Sciences, Poznan, Poland

Abstract: PNH is a rare clonal disorder of hematopoietic stem cells, therefore all blood cell lineages are involved. The main feature is an increased sensitivity of erythrocytes to complement-mediated cell lysis due to deficiency of membrane-bound GPI (glycosylphosphatidylinositol)-anchored proteins which normally function as inhibitors of reactive hemolysis. In the present study, we performed flow cytometric analysis using monoclonal antibodies against CD55 and CD59 for the detection of PNH-type clone in the blood of 50 patients (28 females and 22 males, age range 7-67 yrs). In one patient only we found a large population (95%) of granulocytes with decreased expression of both CD55 and CD59 molecules (type I PNH) and in two others with partial loss of CD55 expression (type II PNH). The expression was determined chiefly on granulocytes which in the control group showed reliable and high expression of CD55 and CD59.

Key words: Paroxysmal nocturnal haemoglobinuria - CD55 - CD59 - DAF - MIRL - Haemolytic anaemia - Flow cytometry

Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare acquired hematological disorder characterized by anemia, intravascular hemolysis, bone marrow hypoplasia, as well as tendency to thrombosis and infection. Increased sensitivity of erythrocytes to complement-mediated cell lysis is due to deficiency of membrane-bound GPI (glycosylphosphatidylinositol)-anchored proteins which normally function as the inhibitors of reactive hemolysis [7, 17]. GPI-anchored proteins include: complement regulatory proteins like DAF (decay accelerating factor, CD55), MIRL (membrane inhibitor of reactive lysis, CD59), and other membrane proteins participating in immune function (CD14, CD16, CD24, CD48, CD58, CD66b, CD67) [4, 12, 13, 15]. PNH is a clonal disorder of hematopoietic stem cells and all blood cell lineages are involved. Disturbances of GPI-anchored proteins are commonly due to somatic mutation of the phosphatidylinositol glycan complementation class A (PIG-A) gene [9]. The mutation can be either spontaneous or facilitated by some conditions [7]. The coexistence of multiple clones with different mutations of PIG-A gene in the same PNH patient has been also reported. Apart from mutation there is also a survival advantage of the PNH clone [1,10]. Dominance of PNH clone is probably due to negative selection against the non-mutated cells rather than positive selection of the PIG-A gene mutant cells [3, 10]. In PNH patients, usually coexist normal and deficient subpopulations of cells with regard to GPI-anchored protein expression. PNH-phenotype has been also described in various hematological disorders, such as aplastic anaemia, myelodysplastic syndromes, myeloproliferative and lymphoproliferative syndromes like acute non-lymphoblastic leukemias, non-Hodgkin’s lymphomas, Hodgkin’s disease and acute lymphoblastic leukemias [10, 12, 15, 18]. Various tests are used for the determination of PNH. Routine diagnostics involves: the Ham’s test (acidified serum lysis test) and the sugar water yeast (sucrose hemolysis) test but both determine only the sensitivity of red blood cells to complement-mediated lysis. For the evaluation of reduced expression of GPI-anchored proteins on all blood cells, flow cytometric analysis is probably the best method to date [13,14]. In the present study, using monoclonal antibodies against CD55 and CD59 and flow cytometry, we searched for PNH-type clone in the blood of patients with clinical symptoms suggesting PNH.
Materials and methods

Patients. We studied 50 patients (28 females, 22 males, aged 7 to 67 years) clinically suspected for PNH, diagnosed at the Department of Haematological and Haemopoietic Proliferative Diseases and Department of Child Infectious Diseases of University of Medical Sciences, Poznan, Poland. This group clinically manifested hemolytic anaemia, in some patients accompanied by hematuria, abdominal pain and signs of persistent infection, most commonly of the urogenital tract. Five control subjects were healthy (blood donors).

Detection of PNH-type cells. Five milliliters of peripheral blood was collected from antecubital vein into a tube with EDTA anticoagulant. One-hundred microliter blood samples were immunostained in tubes with the following two-color combinations of monoclonal antibodies (Becton Dickinson); isotype control mAbs and anti-CD45 FITC/CD14 PE, anti-CD3 FITC/CD19 PE, anti-CD4 FITC/CD8 PE, anti-CD3 FITC/CD16+56 PE mAbs for determination of main sub-populations of leukocytes.

For the detection of PNH-type granulocytes, monocytes and lymphocytes we used fluorescein-isothiocyanate (FITC)-labeled anti-CD55 (clone IA10) and phycoerythrin (PE)-labeled anti-CD59 (clone p282, H19) monoclonal antibodies (Becton Dickinson). The cells were incubated with antibodies at room temperature for 15 min in the dark, then a 2-mL volume of FACS-lysing solution (Becton Dickinson) diluted 1:10 was added to each tube, gently mixed, and incubated for further 10 min in the dark. The tubes were washed twice with PBS and centrifuged at 1200 g for 4 min. The white cell pellets were resuspended in 0.5 mL PBS and analyzed by flow cytometry using a FACScan flow analyzer and CellQuest software (Becton Dickinson). At least 20 × 10³ events were collected. Expression of CD55 and CD59 molecules was quantified by the determination of mean fluorescence intensity (MFI).

Results and discussion

In the group of patients with the suspicion of PNH, alterations of expression of CD55 and CD59 were found in 3 patients only. In two patients, partial depletion of the above-mentioned markers (so-called type II, Fig. 1) was established, while in one a total loss of both antigens was evident (so-called type I, Fig. 2) [14]. The criterion of recognition of cell clone showing absence of both markers was the detection of cell subset (above 5% of cells) in which there was a fall of expression of both antigens, at least 1 log MFI. Population of phagocytes (neutrophils and monocytes) was considered as the most suitable for the evaluation of CD55 and CD59 expression. For cytometric analysis only such cells were selected which in terms of FSC/SSC (size, cell granularity) showed features of living, undamaged and not apoptotic cells. In the control group (healthy blood donors), there was no alteration in the expression of CD55 and CD59 on phagocytic cells (Fig. 3).

The results of this study show that PNH is rather rare disorder in spite of clinical suspicions. We were able to confirm PNH diagnosis in only 3 patients out of 50 tested, what corresponded to 6% of cases.

We analyzed the expression of two GPI-anchored proteins CD55 and CD59. Both are regarded as the most valuable diagnostic markers of PNH [6, 12, 19]. CD59 is probably the most important protein, protecting the cell from the complement-dependent lysis [17].

According to Hsi [8], it is necessary to evaluate both markers because the assessment of only one antigen (CD59) provides false positive results in blood donors. Other members of GPI complex such as CD67, CD48, CD24 and CD16 are also considered as markers of PNH [17], but their use for detection of the disease-specific cell clone is less common.

Expression of CD55 and CD59 may be determined on all blood cells such as leukocytes, erythrocytes and platelets [17]. We have chosen the population of phagocytes (both granulocytes and monocytes) as relatively durable during laboratory processing. Erythrocytes showing deficit of GPI proteins are known to be hemolyzed easily, what makes the detection of searched clone difficult [12, 13]. Alternatively one can assess reticulocytes, because this cell subset is usually increased in patients with PNH and shows higher resistance to hemolysis than mature red blood cells [4]. According to Őelschlagel et al. [12], the optimal approach appears to be a simultaneous evaluation of both erythrocytes and granulocytes for the expression of CD55 and CD59. This is possible with the use of commercial kits REDQUANT and CELLQUANT for assessment of the respective cell subsets.

Detection of the cell clone possessing PNH phenotype may be facilitated by the application of monoclonal antibodies against CD11b and glycoporphin in concert with CD55 and CD59. This approach provides better gating of the appropriate subpopulations [18]. Another possibility for better definition of negative clones could be to determine separate cell subsets based on Laser Scanning Cytometry, new technology combining capabilities of flow cytometry and morphology [5] or to assess distribution and density of target molecules [16]. In this study, the presence of negative cell population above 5% was adopted as a criterion for recognition of the PNH cell clone. Cell acquisition was 20 × 10³. According to Wang et al. [18], using sensitive flow cytometry and acquisition of 10³ cells one can accept the presence of >0.003% cells within CD11b+ cells (granulocytes) as a PNH clone. It may explain confirmation of PNH diagnosis in three patients only within the cohort of 50 patients. Such low number of positive cases may be also due to in vivo lysis of cells showing altered expression of GPI complex and problems with the detection of single cells.

It is worth to remember that the clone with features of PNH can be found in the blood of patients with myelodysplastic syndromes. This is manifested by lower tendency to progression towards acute leukemia, less karyotypic
**Fig. 1.** Scattergram A shows partial loss of CD59 expression on backgated (scattergram B) monocytes and granulocytes in patient with type II PNH. Scattergram C represents isotype control.

**Fig. 2.** Scattergram A shows almost absence of CD55 and CD59 expression on backgated (scattergram B) monocytes and granulocytes in patient with type I PNH. Scattergram C represents isotype control.

**Fig. 3.** In comparison to PNH, monocytes and granulocytes (scattergram B) show uniform and full expression of CD55 and CD59 antigens on all cells in healthy individual. Scattergram C represents isotype control.
disturbances and better response for cyclosporin treat-
m ent. It is supposed that the presence of PNH clone in
the course of myelodysplastic syndrome suggests milder
damage of the bone marrow [7].

To summarize, cytometric evaluation of CD55 and
CD59 antigens in the population of blood granulocy-
tes/monocytes permits rapid confirmation of diagnosis
of paroxysmal nocturnal hemoglobinuria, at least in
those patients in which complement-dependent cell lysis
is relatively low. Moreover, this assay may add to mo-
nitoring and prognosis in patients with myelo- and lymphoproliferative disorders.

References

Dynamics of hematopoiesis in paroxysmal nocturnal hemoglo-
binuria (PNH): no evidence for intrinsic growth advantage of
PNH clones. Leukemia 16: 2243-2248

mutations and cellular selection in paroxysmal nocturnal hae-
moglobinuria. Lancet 343: 951-953

Superior growth of glycoprophosphatidylinositol-anchored prote-
in-deficient progenitor cells in vitro is due to the higher apopto-
tic rate of progenitors with normal phenotype in vivo. Exp
Hematol 30: 774-782

peripheral erythroid for CD59 in paroxysmal nocturnal haemo-

cal capabilities of laser scanning cytometry (LSC) that comple-
ment flow cytometry. Folia Histochem Cytobiol 39: 87-91

Orfao A (2002) Comparative analysis of different flow cytome-
try-based immunophenotypic methods for the analysis of CD59
and CD55 expression on major peripheral blood cell subsets.
Cytometry 50: 191-201

Kagimoto T, Mitsuya H, Kagimoto T, Mitsu H, Nakakuma H (2002) Frequent detec-
tion of T cells mutations of the hypoxanthine-guanine phospho-

flow cytometry. Evaluation of the REDQUANT and CELLQU-
ANT kits. Am J Clin Pathol 114: 798-806

(2000) PNH cells are as sensitive to T-cell-mediated lysis as
their normal counterparts: implications for the pathogenesis
of paroxysmal nocturnal haemoglobinuria. Br J Haematol 111:
1158-1163

logy of paroxysmal nocturnal hemoglobinuria. Med Sci Monit
9: 161-172

Konninaka Y, Anagyrou K, Korovesis K, Mavrogianni D,
Varni E, Viniou N, Konstantopoulos K (2002) Red cells with
paroxysmal nocturnal hemoglobinuria-phenotype in patients
with acute leukemia. Hematology 7: 69-74

cytometric method for screening paroxysmal nocturnal haemo-
globinuria (PNH) measuring CD55 and CD59 expression on

of glycosylphosphatidylinositol-anchored proteins to assess
paroxysmal nocturnal hemoglobinuria clone size. Cytometry
42: 234-238

hocyte subset analysis and glycosylphosphatidylinositol pheno-
type in patients with paroxysmal nocturnal hemoglobinuria.
Blood 92: 1799-1806

for paroxysmal nocturnal hemoglobinuria (PNH) clone in Egyp-

(2001) Confocal microscopy: principles and applications to the
field of reproductive biology. Folia Histochem Cytobiol 39:
75-86

proteins in paroxysmal nocturnal haemoglobinuria. Blood
7: 75-86

Clinical significance of a minor population of paroxysmal noc-
turnal hemoglobinuria-type cells in bone marrow failure syn-
drome. Blood 100: 3897-3902

He G, Chu Y, Yang T (2002) Clinical analysis of 78 cases of
paroxysmal nocturnal hemoglobinuria diagnosed in the past ten

Received: December 3, 2003
Accepted after revision: December 15, 2004