

Frequency of paroxysmal nocturnal hemoglobinuria in patients attended in Belém, Pará, Brazil

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Background: Paroxysmal nocturnal hemoglobinuria is a hematological disease with complex physiopathology. It is genetically characterized by a somatic mutation in the PIG-A gene (phosphatidylinositol glycan anchor biosynthesis, class A), in which the best known antigens are DAF (decay accelerating factor or CD55) and MIRL (membrane inhibitor of reactive lysis or CD59).

Objective: To determine the frequency of paroxysmal nocturnal hemoglobinuria in patients attended at the HEMOPA foundation from November 2008 to July 2009.

Method: Thirty patients, with ages ranging from two to 79 years old and suspected of having paroxysmal nocturnal hemoglobinuria were examined. All patients were immunophenotyped by flow cytometry for the CD5, CD59, CD16 and CD45 antigens.

Results: Paroxysmal nocturnal hemoglobinuria was identified in nine of the thirty patients investigated. Another 3 cases had inconclusive results with CD59-negative labeling only for neutrophils. The highest frequency of paroxysmal nocturnal hemoglobinuria patients (7/9) and inconclusive cases (2/3) were between 19 years old and 48 years old, with a median of 28 years.

Conclusion: These results show the importance of flow cytometry to identify cases in which patients are deficient in only one antigen (CD59).

Keywords: Immunophenotyping; Flow cytometry; Paroxysmal nocturnal hemoglobinuria; Diagnosis; Antigen, CD55; Antigen, CD59

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH), first described in 1882, is a hematological disorder of complex physiopathology which leads to multiple clinical disorders and complications. Its exact incidence is unknown but it equally affects both genders and occurs at any age, but mainly in young adults with a mean age of 35 years old.^(1,2)

PNH is a clonal, acquired and rare disease, which is characterized genetically by a somatic mutation in the PIG-A gene (phosphatidylinositol glycan anchor biosynthesis, class A), located on the short arm of chromosome X (Xp22.1). The molecular alterations of this gene are heterogeneous with more than 180 mutations having been described in patients with PNH; the majority of these result from small insertions or deletions of one or two nucleotides, which cause alterations and block gene transcription and where the production of the gene product is null. Point mutations can also occur producing a partially functional molecule.⁽³⁻⁶⁾

This defect results in a deficiency in the biosynthesis of glycosylphosphatidylinositol (GPI) which is responsible for the anchorage and fixation of the regulatory antigens of the complement system (CS), CD55 antigen or decay accelerating factor (DAF) and CD59 antigen or membrane inhibitor of reactive lysis (MIRL), on the outer surface of erythrocyte, leukocyte and platelet membranes. This makes these cells vulnerable to lysis mediated by complement which results in hemolytic anemia, a certain degree of bone marrow insufficiency and the release of large amounts of free hemoglobin into the plasma, which can lead to an increase in the consumption of nitric oxide and clinical manifestations that include fatigue, abdominal pain, esophageal spasms, erectile dysfunction and various degrees of thrombosis (classic or subclinical PNH).^(1,7,8)

In some special situations, PNH can be associated with other specific disorders of the bone marrow such as aplastic anemia, myelodysplastic syndrome or other myelopathies including myelofibrosis.⁽⁹⁻¹²⁾

Estimated survival for untreated patients with classic or subclinical PNH is approximately 8 years with the main causes of death being thrombotic complications and

progressive pancytopenia. Rarely, spontaneous remission occurs (10% to 15% of cases) or transformation to acute myeloid leukemia.^(3,9,13,14)

The diagnosis of PNH is based on clinical findings and laboratory tests that detect GPI-binding to red cell membrane proteins or that demonstrate the presence of red blood cells that are exceptionally sensitive to hemolysis by the complement system. These tests include the acidified serum test (Ham's test), determination of the CD55 and CD59 antigens using gel column technology or immunophenotyping by flow cytometry. The last test is the most sensitive, specific and precise in the diagnosis of PNH, because it identifies this defect in red blood cells, granulocytes and platelets and shows the stage of the disease based on the quantification of PNH cells.^(5,11,12)

Anti-CD55 and anti-CD59, the most commonly tested monoclonal antibodies in the diagnosis of PNH by immunophenotyping using flow cytometry, are sufficient for a definitive diagnosis. However, a deficiency of GPI can also be detected by testing these monoclonal antibodies along with anti-CD58, anti-CD14, anti-CD24, anti-CD48, and anti-CD16, among others.^(7,10,15)

Thus, the objective of this study was to implement a diagnostic service for PNH using flow cytometry and to define the frequency of this disease among patients suspected of having PNH.

Methods

Patients

In the period from November 2008 to July 2009, a study was carried out of 30 patients with ages between 2 and 79 years, suspected of having PNH. These patients were attended at the HEMOPA Foundation and referred by the attending physician to the flow cytometry laboratory of the HEMOPA Foundation for immunophenotyping by flow cytometry of anti-CD55 and anti-CD59 antibodies.

Biological samples

In this descriptive, cross-sectional study, peripheral blood samples from the patients were collected in hemolysis tubes with 0.5 mL EDTA. The patients underwent further diagnostic evaluations for PNH.

Immunophenotyping

Each Falcon tube was first labeled for either red blood cells or neutrophils of suspected (patient) and control (normal) blood samples. In the analysis of red blood cells and neutrophils, 50 µL and 100 µL of peripheral blood was added to tubes, respectively. An additional volume of 95 µL PBS diluted 10 times was added to each tube. Five microliters of the CD55, CD59, CD16 and CD45 antibodies (labeled with FITC, PE and Pe-Cy5 fluorochromes) were added to each tube, and the tubes were vortexed and incubated for 30 minutes in the dark. For neutrophil analysis, 100 µL of the

first Lysis Solution (DAKO) were added, and the tubes were vortexed and incubated for ten minutes in the dark. Next, 1 mL of the second Lysis Solution (DAKO) was added, and the tubes were vortexed and incubated for twenty minutes in the dark. At the end of the incubation period, tubes both for the analysis of red blood cells and for neutrophils were centrifuged at 700 rpm for five minutes and the supernatants were discarded. Three mL and 1 mL of PBS diluted ten times were added to the tubes for red cell and neutrophils analysis, respectively and the tubes were vortexed. Subsequently, 10,000 cells were separated and analyzed for three colors in a FACS Calibur flow cytometer, with Cell Quest Pro (Becton Dickinson, San Jose, CA).

Statistical analysis

Descriptive and nonparametric methods were employed to statistically analyze the incidence of PNH in the patients with a p-value ≤ 0.05 being considered significant.

Results

Of the total of 30 patients studied, nine had positive diagnoses for PNH and 18 had negative results. In three cases, it was not possible to arrive at a conclusive diagnosis, as on staining for CD59 in neutrophils, the patients showed cells that were partially negative, but showed normal staining for CD55 and CD59 in red blood cells.

The majority of the patients with PNH (7/9) or with inconclusive diagnosis (2/3) were between 19 years and 48 years old with a median age of 28 years. The highest number of positive cases (3/9) occurred in the 21 to 30 age range. There was no significant difference for PNH positive cases between genders with four positive cases in males and five in females.

Discussion

One of the main causes of complications in PNH, is the delay in establishing a correct diagnosis and discarding myelodysplastic syndromes in view of the many similarities in the clinical signs and laboratory results of patients with these diseases.⁽⁹⁻¹¹⁾

As tests for PNH do not exist yet in several state capitals of North Brazil, it is necessary to refer patients with PNH to other centers that provide this service.

It became possible to precisely identify PNH patients only recently in Pará, after the implantation of immunophenotyping by flow cytometry at the HEMOPA Foundation.

This study shows that despite the small sample size, 1) there was no statistical difference in respect to gender and 2) the majority of cases showing positivity for PNH were between 19 and 48 years old, with a median age of 28 years. These findings are similar to those reported in the literature.^(11,12)

Bessler et al.⁽¹⁾ reported that the diagnosis of PNH is more often made in young adults with a median age of 35 years, but that PNH also occurs in the elderly and children and affects both genders equally.

Despite of not having been one of the objectives of this work, it is important to comment that some hypotheses could explain the occurrence of the three inconclusive cases: the presence of small clonal populations of PNH cells masked by normal cells from transfusions within the three months prior to the tests;^(5,7,11-13,16,17) small populations of granulocytes without glycosylphosphatidylinositol-anchored proteins in patients with aplastic anemia or myelodysplastic syndrome but without evidence of hemolysis or other presentations of PNH, with the exception of altered hematopoietic production, and cross reactions with auto-antibodies for other autoimmune diseases such as systemic lupus erythematosus,^(3,9,10,12) as was observed in one of the inconclusive cases presented in this study and only diagnosed correctly as negative for PNH after the suspension of treatment with prednisone.

Conclusion

Thus, our results confirm the importance of the use of flow cytometry and its relevance for the identification of PNH in which the patient is deficient in at least one antigen (CD59).

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