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Short Report

Evaluation of Lymphocyte Subpopulations and Antiphospholipid Antibodies in Patients with Paroxysmal Nocturnal Hemoglobinuria

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Abstract

Several studies have reported an involvement of the immune system in patients with paroxysmal nocturnal hemoglobinuria (PNH) and the presence of an oligoclonal T-cell expansion has been observed.

Nineteen patients (7 males, 12 females; mean age 43.1 years, range: 27-63) with a diagnosis of PNH (Median time from diagnosis 14.8 years, range 6-30) and 40 healthy subjects, 20 males and 20 females (mean age 42.8 years, range 25-62) as control group, were tested. Hemocromocytometric parameters, lymphocyte subpopulations and antiphospholipid antibodies were evaluated.

Patients with PNH had significantly reduced levels of hemoglobin (9.8±3 gr/dL vs 14.8±1 gr/dL, p <0.001), white blood cells (3,747.9±1,915 µL vs 6,239.4±1,520 µL, p<0.001), lymphocytes (1,170.2±546 µL vs 2,069.5±482 µL, p <0.001) and platelets (142.3±98.8 x10^3/µL vs 239±56 x10^3/µL, p <0.001) compared to normal controls. The absolute number of CD19+ B cells and NK cells was significantly reduced in PNH patients compared to the control group: 60.9±32/µL vs 187.8±91 µL, p <0.001, and 86.3±83/µL vs 273.6±147/µL, p = 0.012, respectively.

No differences were found on hemocromocytometric parameters and lymphocyte subpopulations between the nine patients receiving eculizumab treatment and the ten patients who did not receive the monoclonal antibody.

Four out of 19 (21.05%) PNH patients had antiphospholipid antibodies.

Significant modifications of the hemocromocytometric parameters and lymphocyte subpopulations were found in patients with PNH. The antiphospholipid antibodies may be present in patient with PNH.

Keywords: Paroxysmal Nocturnal Hemoglobinuria; Lymphocyte Subpopulations; Hemocromocytometric Parameters; Antiphospholipid Antibodies

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disorder of hematopoietic cells characterized by the triad of hemolytic anemia, high risk of venous thrombosis and cytopenias. This is a result of the expansion of a stem cell clone with an acquired mutation in the PIG-A gene [1], resulting in the defective synthesis of glycosylphosphatidylinositol (GPI) and, therefore, in a deficiency on the surface of circulating blood cells of all GPI-linked proteins. Since two complement inhibitors, CD55 and CD59, are GPI-linked, this results in intravascular complement-mediated lysis of red cells [2,3]. The presence of an oligoclonal T-cell expansion has been reported in patients with PNH, aplastic anemia and myelodisplastic syndrome [4,5]. These data support the hypothesis of an autoimmune-mediated mechanism playing a role in the bone marrow failure occurring in patients carrying with a PNH clone [6]. Moreover, an increase in T cells expressing the activating NK-cell receptors KIR2DS4, NKG2C and NKG2D with cytotoxic activity towards hematopoietic progenitor cell lines has been described in PNH patients, further supporting a possible role of the host immune system in the bone marrow failure and clonal expansion in PNH [7]. The presence of antiphospholipid antibodies has also been observed in PNH patients both with and without thrombotic complications [8,9]. Moreover, immunosuppressive treatment may be effective in some patients with PNH and these evidences strengthen the hypothesis of an involvement of the host immune system in patients with PNH [10,11]. In this study we evaluated hemocromocytometric parameters and lymphocyte subpopulations in patients with a diagnosis of PNH and in normal controls in order to evaluate potential modifications of these parameters related to the disease.

Materials and Methods

Nineteen patients (7 males, 12 females; mean age 43.1 years, range: 27-63) with a diagnosis of PNH were evaluated for the presence of the PNH clone, lymphocyte subpopulations and antiphospholipid antibodies. The diagnosis of PNH required a positive HAM test confirmed by flow cytometric studies of the expression of GPI-linked proteins on erythrocytes and white blood cells (WBCs) using specific monoclonal antibodies (mAbs)[12]. The median time from diagnosis of PNH to the analysis was 14.8 years (range 6-30). Nine of the 19 patients (47.36%) with PNH had undergone treatment with the monoclonal antibody eculizumab. Nine patients received eculizumab for persistent severe anemia and one for anemia and thrombotic complication. The median duration of treatment was 3.0 years, (range 1.6-7.6).

As control groups, 40 healthy subjects, 20 males and 20 females, aged between 25 and 62 years (mean age 42.8 years), were evaluated for the same parameters.

A comparison of hemocromocytometric parameters and lymphocyte subpopulations was also performed between the nine patients receiving eculizumab treatment and the 10 patients who did not receive the monoclonal antibody, in order to evaluate potential effect of the therapy on these parameters.

The phenotypic analysis was performed on the FACSCanto I flow cytometer (Becton Dickinson, San José, CA) using two mAb combinations for granulocyte and monocyte populations, one for RBC analysis (Table 1) and two for the lymphocyte subpopulations (B, T, NK, T-NK). For routine purposes, collection of 30,000 cells of interest (on the granulocyte, monocyte and erythrocyte gates) was sufficient to detect populations at sensitivity of 1%, or more. All antibodies were from BD Biosciences (Becton Dickinson) with the exception of FLAER (Cedarlane, Burlington, Canada) and CD59 MEM-43 (Invitrogen, Carlsbab, CA).

Cell populations	FITC	PE	PerCP	PE-Cy7	APC	APC-Cy7
Granulocytes and monocytes	FLAER (or CD66b)	CD24	CD45	CD33	CD15	CD14
RBCs	GPA-A	CD59	CD45			
Lymphocytes	CD16	CD56	CD3	CD45	CD4	CD8
	CD20	CD5	HLA-DR	CD45	CD19	CD3

Table 1. Monoclonal antibodies combinations to determine PNH-cells and the residual lymphocytic population in each sample.
Gating strategy: We used the following sequences of analysis: CD15 vs CD33; CD45 vs SSC; FSC vs SSC (granulocytes). CD45 vs SSC; CD33 vs SSC; FSC vs SSC (monocytes). FSC-log vs SSC-log; GPA vs SSC (RBCs). CD45 vs SSC; FSC vs SSC (lymphocytes).

A detailed immunophenotypic characterization of the expression of the above-mentioned GPI-linked proteins was performed with either a stain-no-lyse-no-wash direct immunofluorescence technique for RBCs or a stain-lyse-and-then-wash direct immunofluorescence approach for the different WBC subsets (granulocyte and monocyte populations). Flow cytometric data were analyzed with the FACSDiva software (BD). Monocytes, granulocytes and RBCs were easily distinguished using side scatter (SSC) vs forward scatter (FSC) and CD45 vs SSC analysis. Moreover, for a most accurate gating, lineage markers, such as bright CD15 for granulocytes, bright CD33 for monocytes and negative CD45/positive Glycophorin-A (GPA) for RBCs were employed.

According to their relative fluorescence intensity, flow cytometric analysis allowed to identify and quantify, in a dot plot histogram, cells completely deficient of GPI-anchored proteins (PNH-III) or partially deficient (PNH-II) and to distinguish

them from normal cells (PNH-I). Moreover, it was possible to analyze a simultaneous loss of FLAER plus CD24 and CD66-b, FLAER plus CD14 and CD59 on granuloctyes (gated as CD15 high, CD33 low and high SSC), monocytes (gated as CD33 high, CD15 low and lower SSC) and erythrocyte cells (gated as CD45 negative and GPA positive), respectively.

The kaolin clotting time (KCT) was performed according to the method of Exner et al [13] using a 2% kaolin suspension. Mixing studies, aimed at documenting the inhibitory activity of prolonged KCT samples, were performed by adding normal PPP in a proportion of 1:1 and 1:4 in all KCT prolonged samples. Only samples with a prolonged KCT not corrected by mixing procedures and characterized by a correction of KCT after the addition of natural phospholipids were considered positive for the presence of Lupus Anticoagulant (LA). The normal range (mean ± 2SD) of the KCT ratio was 0.74-1.30.

Also Silica Clotting Time (SCT) was used to detect the presence of LA with the same procedures of mixing and correction used for KCT test (Normal limit, Ratio < 1.26) [14].

dRVVT was carried out according to the method of Thiagarajian et al [15] using a commercial kit (Instrumentation Laboratory Diagnostics, Milan, Italy) and following the manufacturer's instructions. The normal range (mean ratio ± 2SD) of dRVVT was 0.85 - 1.25. The normal ranges of KCT and dRVVT ratio were obtained by testing 80 healthy volunteers (40 males and 40 females, mean age 42.8 years, range 24-65).

IgM and IgG ACA were performed using an ELISA method [16] using a commercial kit (Chromogenix, Molndal, Sweden), according to the manufacturer's instruction. Results were expressed as GPL units/mL and MPL units/mL (U/mL) according to Harris et al [17]. Values exceeding 13 GPL U/mL and 11 MPL U/mL were considered above the normal limit. According to the International Consensus Statement on an update of the classification criteria to define an APA syndrome, the positivity of APA was defined as the presence in the serum or plasma of lupus anticoagulant or ACA at a medium or high titer [18]. The IgG and IgM isotypes were categorized as positive when >23 GPL U/mL or >11 MPL U/ml, respectively.

The values reported represent the mean of two tests obtained in each patient at least 3 months apart. Seven days before the evaluation of the coagulation parameters the treatment with vitamin k antagonist was discontinued in all patients in order to obtain a normalization of the International Normalized Ratio (INR).

Statistical Analysis

Data were analyzed by the statistical software STATPAL (Statistical package, version 5.0, by Bruce J. Chalmer and David G. Whitmore). The evaluated parameters have been reported as mean ± SD; the lymphocyte count and lymphocyte subpopulations were evaluated also as percentage.

Differences among patients and controls for hemocromocytometric parameters and lymphocyte subpopulations were evaluated both by parametric (Grouped T-test) and non-parametric (Wilcoxon sum rank test) test. The Pearson's r test was used for correlation analysis. Significance was set at $p < 0.05$.

Results

The results of the parameters evaluated are reported in Table 2. PNH patients showed significantly reduced levels of hemoglobin, platelets, WBCs and lymphocyte count in comparison with normal controls.

Analysis of the lymphocyte subpopulations showed a significant reduction of CD19+ and NK cells (CD3-/CD56+/CD16+) in PNH patients in comparison to normal controls, both as percent and absolute number. CD3+ and CD8+ T cells were reduced as absolute number in PNH patients compared to the control group. The same subpopulations were increased, when evaluated as percentage of the whole lymphocytes population in PNH patients versus normal controls. T-NK cells (CD3+/CD56+/CD16-) were not different in PNH patients respect to healthy controls as absolute number, but they were increased in PNH patients as percentage.

The PNH clone size evaluated both on neutrophil granulocytes and monocytes was 73.5±27% (range: 7-99) and 72.3±28% (range: 6-99), respectively. Eighteen patients showed only the PNH III clone; one showed both the PNH II and PNH III populations.

Four out of 19 (21.05%) PNH patients had antiphospholipid antibodies while no out of the 40 normal controls (0%) had antiphospholipid antibodies (Exact Fisher Test, $p = 0.0085$): two patients showed LA positivity (KCT Ratio = 1.50 and 2.38; SCT Ratio = 1.54 and 2.35, respectively), and two had anticardiolipin antibodies (26 GPL U/mL and 24 GPL U/mL, respectively).

The evaluation of lymphocyte subpopulations and hemocromocytometric parameters in PNH-APA positive patients (N=4) and in PNH patients without APA (N=15) showed a significant lower lymphocyte count in patients with APA (717.5±298 μL vs 1,292±539 μL, p=0.02). The total WBC count was not different between the two groups (2,757.5±1,003 μL vs 4,012.1±2,036 μL, p=ns). The PNH clone, evaluated on neutrophils (77.7±17% vs 63.4±36%, p=ns) and monocytes (76.37±17.5% vs 64.78±17.5%, p=ns) was not different between the two groups of patients. A significant inverse correlation was found between the PNH+ clone and hemoglobin levels (Pearson's r test = -0.62, p=0.003) in all PNH patients.

Table 2

Comparison of lymphocyte subpopulations and hemocromocytometric parameters between patients with PNH and normal controls.

	PNH patients (n=19)	Normal controls (n=40)		
	Mean±SD (µL) (%)	Mean±SD (µL) (%)	p*	p**
Lymphocytes	1,170.2±546 (34.1±13)	2,069.5±482 (33.6±6)	<0.001 ns	<0.001 ns
CD19	60.9±32 (5.2±2)	187.8±91 (8.9±3)	<0.001 <0.001	<0.001 <0.001
CD3	989.9±482 (84.0±6)	1,489.1±418 (71.7±7)	<0.001 <0.001	<0.001 <0.001
CD8	467.6±270 (39.3±9)	656.2±268 (30.9±8)	0.012 <0.001	0.015 0.001
T-NK	98.3±72 (8.8±6)	102.1±82 (4.6±4)	ns 0.012	ns 0.013
NK	86.3±83 (6.4±5)	273.6±147 (13.7±8)	<0.001 <0.001	<0.001 <0.001
T4	540.7±242 (45.4±8)	902.7±204 (44.4±7)	<0.001 ns	<0.001 ns
T8	430.1±225 (35.3±8)	527.9±260 (24.8±9)	ns <0.001	ns <0.001
Hemoglobin (Gr/dL)	9..8±3	14.8 ±1	<0.001	<0.001
White blood cells	3,747.9±1,915	6,239.4±1,520	<0.001	<0.001
Platelets (x 10^3/µL)	142.3±98.8	239±56	<0.001	<0.001

*Grouped T-Test, ** Wilcoxon Sum Rank Test. ns = not significant
PNH = Paroxysmal Nocturnal Hemoglobinuria, SD = Standard Deviation

Table 3

Comparison of lymphocyte subpopulations and hemocromocytometric parameters between PNH patients who received or not eculizumab treatment.

	PNH patients (n=9) receiving ecoluzimab	PNH patients (n=10) not receiving eculizumab		
	Mean±SD (µL) (%)	Mean±SD (µL) (%)	p*	p**
Lymphocytes	1,017.7±573 (35.6±12.9)	1.309±510 (32.7±12.8)	ns ns	ns ns
CD19	59.7±40 (5.3±2)	62±25 (5±2)	ns ns	ns ns
CD3	870.4±508 (83.2±5)	1097.5±457 (84.7±6	ns ns	ns ns
CD8	402.5±216 (37.9±9)	526.1±310 (40.6±8)	ns ns	ns ns
T-NK	72.5±69 (9.1±6)	121.2±71 (8.7±6)	ns ns	ns ns
NK	65.1±72 (6.3±4)	107.4±92 (6.6±5)	ns ns	ns ns
T4	476.5±273 (43.9±9)	598.5±207 (46.7±6)	ns ns	ns ns
T8	369.7±196 (34.8±8)	484.5±245 (35.8±8)	ns ns	ns ns
Hemoglobin (Gr/dL)	8.6±2	10.8 ±3	ns	ns
White blood cells	3,025.5±1,545	4,398±2,055	ns	ns
Platelets (x 10^3/µL)	119.2±105	163.1±93	ns	ns

*Grouped T-Test, ** Wilcoxon Sum Rank Test. ns = not significant
PNH = Paroxysmal Nocturnal Hemoglobinuria, SD = Standard Deviation

As to the 9 patients treated with the monoclonal antibody eculizumab, 4 obtained a complete response with a normalization of the hemoglobin levels and no further need of packed red cell transfusions, 1 a partial response with a reduction of packed red cell transfusion requirement, and while 4 did not respond to treatment.

No differences were found on hemocromocytometric parameters and lymphocyte subpopulations between the nine patients receiving eculizumab treatment and the ten patients who did not receive the monoclonal antibody (Table 3).

The PNH clone, evaluated on neutrophils and monocytes between the patients receiving eculizumad or not (78.4±21% vs 57.2±38%, p = ns and 76.6±20% vs 56.8±37%, p = ns, respectively), was similar in these two groups of patients.

Discussion

An involvement of the immune system has been hypothesized in patients with PNH and it could play an important role in the mechanism of expansion of the PNH clone by autoimmune-mediated bone marrow failure with a preferential survival of GPI-deficient hematopoietic progenitor cells [5,6]. T cells expressing the NK-cell marker CD56 are increased in patients with PNH and this population is predominantly constituted by differentiated CD8+ T cells [7]. In support of this, peripheral CD56+ T cells are increased also in autoimmune diseases such as rheumatoid arthritis [19], Behcet's disease [20] and sarcoidosis [21].

Peripheral T cells are potent producers of interferon-γ, perforin, granzyme-B and cytokines and play an important role in the regulation of the immune system [22].

We studied the hemocromocytometric parameters and lymphocyte subpopulations in a group of patients with PNH in order to evaluate potential modifications of these cells that play a key role in the function of the immune system. A significant reduction of total WBC, lymphocyte, hemoglobin and platelet count was observed in our PNH patients in comparison with normal controls. The occurrence of aplastic anemia is of frequent observation in these patients [23]. Therefore, hemocromocytometric parameters and lymphocyte subpopulations have been evaluated both as absolute number and as percentage in lymphocytes gate.

In our study, we could find a modification of the lymphocyte subpopulations in patients with PNH. In fact, CD19+ B cells were significantly reduced in PNH patients in comparison with normal controls. Also NK cells were significantly reduced compared to normal controls. No increase was observed in the absolute number of CD3+, CD8+ and T-NK cells in PNH patients. However, it is important to note that our PNH patients had a significant reduction of the lymphocyte count compared to normal controls and, therefore, as indicated by the evaluation of percentage, the CD3+, CD8+T and T-NK lymphocyte subpopulations were relatively expanded in these patients.

A similar pattern of antigen expression has been already reported and has been related to an expansion of autoreactive T cells leading to an autoimmune response in patients with PNH and an increased frequency of T lymphocytes producing interferon-γ has been detected in these patients [24]. Moreover, a significantly increased proliferation of CD3+CD48+ lymphocytes from PNH patients with lymphocytopenia in comparison with the CD3+CD48+ healthy control-derived subset has been reported [25]. More recently, an autoreactive CD1d-restricted, GPI-specific T-cell population with a possible role in the mechanism of bone marrow failure has been found expanded in patients with PNH [26].

It is interesting to note that NK cells were reduced in PNH patients compared to normal controls. These results confirm previous data on the reduced number of B and NK cells in PNH [27]. NK cells, as well as other lymphocytes subpopulations, are involved in the PIG-linkage defect of PNH and susceptible to autologous complement [28]. Moreover, the decrease in B, NK and regulatory T cells (T-reg), an altered cytokine profile of invariant NKT cells (NKTi) and the increasing of C-X-C chemokine type 4 receptor (CXCR4) has been reported in PNH patients. And eculizumab could have a role in modifying the immune profile in these patients by correcting the effect of complement hyper-activation on red cells, but also by normalizing complement-dependent alterations of immunological targets [29]. Despite these quantitative and qualitative abnormalities, none of our patients suffered from an excessive number or severity of infections, as previously reported [27].

The presence of APA has been reported in PNH patients with and without thrombotic complications and this positivity has been associated to the presence of immune system alterations in these patients [8,9]. However, we did not find differences in lymphocyte subpopulations and the PNH clone between PNH patients with and without APA, even though PNH patients with APA had a significant reduction of the lymphocyte count.

Also in our patients eculizumab induced rapid and sustained effects through the stabilization of hemoglobin levels in patients with PNH, reducing the need for packed red cell transfusions in a proportion of patients. Eculizumab was also associated with significant improvements in fatigue and health-related quality of life, as reported in several trials [30]. As to potential effect of the treatment with eculizumab on hemocromocytometric parameters and lymphocyte subpopulations we did not observe differences between the nine patients receiving eculizumab and the ten patients who did not receive the monoclonal antibody. A previous study suggested that eculizumab treatment did not modify the number of NK and Treg cells in PNH patients while it could have a role in increasing B lymphocytes and NKT cells in patients with PNH [29]. Also the size of PNH

clone was not different in patients treated with eculizumab suggesting that the correction of complement hyper activation is not able to modify the immune-mediated mechanism involved in the selection and dominance of PNH precursors as previously described [29,31].

Conclusion

This study showed the presence of significant modifications of the hemocromocytometric parameters and lymphocyte subpopulations in patients with PNH. The treatment with the monoclonal antibody eculizumab does not seem able to modify the lymphocyte subpopulations in patients with PNH.

The antiphospholipid antibodies may be present in patients with PNH and they could represent an expression of the immune system involvement in patients with PNH. However, the role of these alterations in the pathogenesis of the disease and in autoreactive-phenomena is complex and it needs to be further clarified in patients with PNH.

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