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Effect of PNH Clone on the Phenotypic Expression of Aplastic Anaemia

Dr Namrata Punit Awasthi MD[1], Dr. Rajesh Kashyap MD[2], Dr Ritu Gupta MD[3]
[1]Additional Professor, Department of Pathology,
Dr. Ram Manohar Lohia Institute of Medical Sciences Lucknow, UP India
[2]Professor, Department of Hematology,
Sanjay Gandhi Postgraduate institute of Medical Sciences Raebareli Road,
Lucknow, UP India
[3]Professor, Department of Laboratory Oncology
All India institute of Medical Sciences New Delhi, India

***Corresponding Author:**

Dr. Rajesh Kashyap MD
Professor
Department of Hematology
Sanjay Gandhi Postgraduate institute of Medical Sciences
Raebareli Road, Lucknow

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ABSTRACT

PNH and aplastic anaemia (AA) both are acquired clonal hematopoietic stem cell disorders. PNH Clones have increasingly been detected in AA patients by flow cytometric (FCM) analysis. The presence of PNH clones is predictive of a good response to immunosuppressive therapy. However, it is not clear whether the presence of PNH Clone modifies the phenotype of AA. This study was carried out to determine the incidence of PNH Clone in our AA patients and evaluate its influence on the phenotypic expression of disease.

Material & Methods

Seventy-two newly diagnosed AA patients were the subjects of this prospective study. The investigations and diagnosis was made as per the guidelines. PNH clone presence was evaluated by determining the expression of CD55 and CD59 antigen on the erythrocytes and leukocytes by FCM. The severity of AA was classified into very severe (VSAA), severe (SAA) and non-severe (NSAA) according to the criteria proposed by Camitta et al. The clinical and laboratory profile of patients with and without PNH clone was compared.

Results

No PNH clone was detected in 43 of the 72 patients (60%) and in the remaining 29 (40%) patients PNH clone was detected either on RBC, granulocytes or monocytes. It was found that the presence of PNH Clone was more frequently associated with NSAA (45%) and SAA (44%) than with VSAA (25%)

Keywords: PNH, Aplastic Anaemia, Flow Cytometry, CD55, CD59

INTRODUCTION

Aplastic anaemia is an acquired bone marrow failure syndrome characterized by peripheral blood pancytopenia associated with hypocellular bone marrow resulting from an immunological attack of hematopoietic stem cells.[1] Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder caused by somatic mutation of the X-linked phosphatidylinositolglycan class A (PIG A) gene resulting in deficient expression of glycosylphosphatidylinositol anchored proteins (GPI-APs).[2] Deficiency of GPI-linked complement regulatory proteins; membrane inhibitor of reactive lysis (MIRL, CD59) and decay accelerating factor (DAF, CD55) are responsible for the marked susceptibility of red blood cells of PNH to the complement mediated hemolysis leading to the characteristic clinical feature of intravascular haemolytic anaemia.[3] Both PNH and AA are closely related acquired hematopoietic stem cell disorders and this strong association has been recognized since

1961.[4] At some stage of disease patient of PNH may become less haemolytic and more pancytopenic. Conversely, a patient of aplastic anaemia may subsequently develop PNH: the term PNH-AA Syndrome has been used to designate such patients.[5] With the recently developed sensitive flow cytometric analysis , GPI-AP deficient blood cells (PNH Clone) have been detected in aplastic anaemia patients even without clinical haemolytic features of PNH.[6] The term PNH-Subclinical (PNH-sc) has been used for them by Parker et al.[7] Remarkably, it is now appreciated that normal individuals harbour small number of bone marrow cells with PIG-A mutations identical to those causing PNH. [8]

The presence of PNH Clone in aplastic anaemia patients depended on the sensitivity of, and cell lineages evaluated by flow cytometric analysis and has varied between 25% and 67%. [6,9]. It has been shown that presence of increased number of PNH type cells is predictive of a good response to immunosuppressive therapy.[10] It has been observed that patients with larger PNH clone size have milder cytopenias and overt hemolysis [11,12] It is however not clearly known whether the presence of PNH Clone modifies the phenotype of aplastic anaemia. The present study was carried to study the incidence of PNH Clone in aplastic anaemia patients at our centre using sensitive flow cytometric technique and to evaluate the effect of presence of PNH Clone on the phenotypic expression of aplastic anaemia.

Material and Methods

Patients

Seventy-two newly diagnosed patients with aplastic anemia were the subjects of this study. None of these patients had received any prior immunosuppressive therapy. The investigations conducted were complete blood counts, biochemical tests for liver and renal function, serum lactate dehydrogenase (LDH) level. bone marrow aspiration and biopsy, Flow cytometry and radiological investigations when indicated. The disease severity was classified into very severe, severe and non-severe aplastic anemia according to the criteria proposed by Camitta et al. [3])

Detection of PNH Clone by flow cytometry

The PNH clone was evaluated both in erythrocytes as well as leukocytes using FCM. Two millilitres of ethylene diamine tetra acetic acid (EDTA)-anticoagulated peripheral blood was collected from each individual, and sample was processed and acquired within 6 hours of sampling. In case of delay in processing, samples were stored at 4oC. For erythrocytes, a single-wash-no-lyse technique was used. Briefly, 10 µL whole blood was diluted in 1 ml of phosphate buffered saline (PBS). 100 µL of this diluted RBCs were taken and incubated with pre-titrated volumes of phycoerythrin(PE)-conjugated monoclonal antibodies (MoAb) CD55 (IA10, Pharmingen, BD Biosciences, San Diego, CA, USA) and CD59 (H 19, Pharmingen, BD Biosciences) in dark at room temperature for 20 minutes, followed by washing with PBS and then re-suspended in 1% paraformaldehyde. For leukocytes, a Stain-Lyse-Wash protocol was used for all the cases. Lysis of RBCs was carried out with an ammonium chloride based reagent before incubation with CD16 (FITC), CD55 (PE) and CD59 (PE).

The acquisition was performed on FACS Canto II system and analysis performed using FACS Diva software (BD Biosciences, USA). A minimum of 5,000 RBC and 5,000 WBC were acquired. In case of leukopenia it was attempted to acquire at least 2000 neutrophils and a maximum number of monocytes as possible. Cell populations were labelled as PNH clone on FCMI when there was evidence of a population of peripheral blood cells (erythrocytes, granulocytes, or preferably both) deficient in GPI-APs. [7] The cut off positivity for PNH was taken as 1%. Based on the mean relative intensity of CD55 or CD59 fluorescence, cells were separated into three discrete populations. Those with an intensity that lay within the normal range (defined as mean ± 2 SD) calculated from normal healthy controls (n=20) were of the PNH I phenotype. Cells with an intensity within the CD55 or CD59-negative area in the flow cytometric profile, defined as the area containing 99.8% of the negative isotype control cells, were of the PNH III phenotype. PNH II cells were found in between negative and normally positive areas. The granulocytes and monocytes were identified by their characteristic light scatter properties on dot-plot scattergrams and evaluated for expression of CD55 and CD59 with reference to the normal control samples processed on the same day or remaining normal granulocytes and monocytes within the same sample (Figure 1).

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Statistical analysis

Descriptive statistical analysis of main characteristics of patients was performed. Categorical and continuous data were presented in frequency (%), mean ± SD. The association between two categorical variables was seen by Student's t- test.P-value < 0.05 was taken as statistically significant. All the statistical analysis was done using SPSS version 16 software.

The study complied with the institute's IRB rules and guidelines. Prior consent was taken from all the patients.

Results

A total of 72 newly diagnosed patients with aplastic anaemia were evaluated. In 43 of the 72 patients (60%) no PNH clone was detected whereas in the remaining 29 (40%) patients a PNH clone was detected either on RBC, granulocytes or monocytes. Based on the presence of PNH cells, we divided these 72 patients into two groups:

Group 1: **Aplastic anaemia without PNH Clone (n=43)**.There were 33 (77%) males and 10 (23%) females with a M: F ratio of 3.3:1. The mean age was 34.9(range 11-75) years.

Group 2: **Aplastic anaemia with PNH Clone (n=29).**There were 14 (48%) males and 15 (52%) females with a M: F ratio of 0.9:1. The mean age was 35.8 (range 15-66) years.

In Group 1, 17 patients (39.5%) had NSAA, 14 (32.5%) had SAA and 12(28%) had VSAA. In Group 2, 14 patients (48%) had NSAA, 11 (38%) had SAA and 4 (14%) had VSAA (Table 1). It was found that the presence of PNH Clone was more frequently associated with NSAA (45%) and SAA (44%) than with VSAA (25%)

The two groups were compared for Hemoglobin (Hb), Total leukocyte count(TLC), Platelet count (Plt), Absolute Neutrophil Count (ANC) and Reticulocyte count at presentation and the respective means of individual variable in the two groups were studied for statistical significance if any(Table 1). Hemoglobin, total leukocyte count, absolute neutrophil count, platelet count, and reticulocyte count of patients with NSAA were compared within the Groups 1 and 2 in an attempt to study any significant difference. Similarly these parameters were compared in patients of SAA and VSAA within the two groups. The results are shown in table 2.It was found that the P value was >0.05 (not significant) when these variables were compared among the two groups except for platelet counts in patients with very severe aplastic anaemia. It was observed that in Group 1, mean Platelet count was 22.0 and in Group 2 it was 13.2 (p=0.006) however this difference was not present when cases of VSAA and SAA were clubbed together (table 4).

The variables were compared among patients with severe and very severe disease (SAA+VSAA) with those of non severe disease (NSAA) in both the groups (Table 3). Further, patients who had VSAA+ SAA were studied in both the groups and compared for the values of Hb, TLC, Plt, Reticulocyte count and ANC (Table 4).

The analysis reveals that in Group 1, statistically significant difference was found in the mean values for TLC, Plt, ANC and Reticulocyte count in patients with NSAA and VSAA+SAA. In Group 2, statistically significant difference was found in mean values for Platelet and ANC only. This may indicate that presence of PNH clone is somehow modifying the disease expression in relation to severity of disease i.e. presence of PNH clone makes the disease less severe. However in cases of severe and very severe disease, PNH clone has no impact on the disease phenotype.

Flow cytometric immunophenotyping of Aplastic anaemia patients with PNH clone (n=29)

PNH clone in RBC was detected in only 4 cases (14%) out of which 3 cases were of non-severe disease whereas PNH clone in leucocytes was detected in 29 cases (100%). Among leucocytes, PNH clone was found in neutrophils in 24 cases and in monocytes in 22 cases. Monocytes were not evaluated in 2 cases. In 7 patients PNH clone was present only in neutrophils and in 5 patients PNH clone was present only in monocytes.

Discussion

PNH and aplastic anaemia are closely related acquired hematopoietic stem cell disorders. PNH frequently occurs during the clinical course of aplastic anaemia including at the recovery phase.[14].With the introduction of sensitive flow cytometric analysis, GPI-AP deficient blood cells or

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PNH Clones have been detected in aplastic anaemia patients even without clinical haemolytic features of PNH[(6)].Among the available methods, Flow Cytometric immunophenotyping is most sensitive, can quantify and delineate PNH cells with differential expression of GPI-anchored proteins.[15]The presence of PNH Clone in aplastic anaemia patients depended on the sensitivity of, and cell lineages involved in the flow cytometric analysis and varied between 25% and 67%.[6,9] A significant increase in the frequency of $CD55^{-}CD59^{-}CD11b^{+}$ granulocytes (>0.003%) was observed in 88.6% patients with untreated AA at diagnosis.[14] In our study, we found the presence of PNH Clone in 40% patients of untreated aplastic anaemia which is consistent with the other studies.[10-12].

We classified our 72 patients with aplastic anaemia into two groups on the basis of presence or absence of PNH Clone and compared the individual haematological parameters. No significant difference was found among the two groups implying that the PNH Clone had no effect on the phenotypic expression of aplastic anaemia. Haemoglobin (Hb), TLC, absolute neutrophil count (ANC), platelet count and reticulocyte count of patients with NSAA, SAA and VSAA were compared within the Groups 1 and 2 in an attempt to study any significant difference. It was found that the difference between the mean values was not statistically significant except for platelet counts. On further categorizing these patients depending on disease severity, it was found that the presence of PNH Clone was more frequently associated with NSAA (45%) and SAA (44%) than with VSAA (25%). Our results indicate that the presence of PNH Clone does not alter the phenotype of patients of aplastic anaemia however it is associated less frequently with VSAA as compared to SAA and NSAA.

We performed the flow cytometric detection of PNH clones on three cell types i.e. RBC, Neutrophils and monocytes and found that PNH clone was detected in very few cases in RBC but in 100% cases in either neutrophils or monocytes or both. Detection of PNH clone in monocytes was important as in 5 cases the clone was detected only in monocytes but not in neutrophils.The clone sizes were in general more in monocytes than in neutrophils but the difference was not significant statistically.

Guidelines for diagnosis and monitoring of PNH disorders by flow cytometry have been published by Borowitz MJ et.al. [16] and Sutherland et. al. [17] Sensitivity for detection of PNH clones for routine analysis has been suggested as 1% which was achieved in the present study. For identifying granulocytes and monocytes CD16 along with scatter properties was utilized in the present study. However erythrocytes were identified based on light scatter only. Another limitation is that FLAER was not used in this study which is perhaps the most useful reagent for detecting PNH clones in WBC.

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LEGENDS

Figure 1: Flow cytometric dot plots showing (A) gating of neutrophils and monocytes based on forward and side scatter; (B & C) two population of neutrophils and monocytes with bright & dim expression of CD16, CD55 & CD59; (D) Histogram with type I, II & III RBC based on expression of CD59

Table 1 CLINICAL AND LABORATORY CHARACTERISTICS OF THE PATIENTS WITH APLASTIC ANEMIA

	Group 1 (n=43) **AA without PNH Clone**	**Group 2 (n=29)** **AA with PNH Clone**	**P value**
Age (years) Mean (Range)	34.9 (11-75)	35.8 (15-66)	
Sex Male:Female ratio	3.3:1	0.9:1	
Hb (g/dl) Mean ±SD	6.2 ±2.9	6.9 ±2.6	0.3
TLC ($\times 10^9$/L) Mean ±SD	2.6 ±1.3	2.7±0.9	0.5
Platelet ($\times 10^9$/L) Mean ±SD	38.1 ±68.2	40.0 ±59.2	0.9
ANC (× cells/ml) Mean ±SD	891.3 ±903.0	985.2 ±914.7	0.6
Reticulocyte (%) Mean ±SD	1.35 ±1.5	1.84 ±1.7	0.2
NSAA **(total n=31)** **N (%)**	17 (39.5%)	14 (48%)	-
SAA **(total n=25)** **N (%)**	14 (32.5%)	11 (38%)	-
VSAA **(n=16)** **N (%)**	12(28%)	4(14%)	-

Abbreviations: SD standard deviation; AA: Aplastic anemia; NSAA: non severe AA; SAA: severe AA; VSAA: very severe AA. PNH: paroxysmal nocturnal hemoglobinuria

Hb: Hemoglobin; TLC: total leukocyte count; ANC: absolute neutrophil count.

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Table 2 Comparison of haematological parameters in patients with NSAA, SAA and VSAA

	NSAA(n=31)	SAA(n=25)	VSAA(n=16)
Hb level (g/dl)			
Mean ±SD			
Group 1	6.0(±3.1)	6.1(±2.9)	6.8(±2.8)
Group 2	7.1(±2.3)	6.6(±3.0)	6.6(±2.7)
(P value)	(0.2)	(0.6)	(0.9)
TLC($\times 10^9$/L)			
Mean ±SD			
Group 1	3.1(±1.4)	2.7(±1.3)	1.7(±0.6)
Group 2	3.1(±0.9)	2.5(±0.9)	2.3(±0.9)
(P value)	(0.9)	(0.6)	(0.3)
Platelet ($\times 10^9$/L)			
Mean ±SD			
Group 1	69.3(±101.8)	14.0(±8.5)	22.0(±8.6)
Group 2	65.5(±78.5)	17.3(±6.0)	13.2(±2.2)
(P value)	(0.9)	(0.2)	**(0.006)**
Reticulocyte count			
(%)	2.3(±1.8)	0.5(±0.5)	0.8(±0.8)
Group 1	2.3(±1.5)	1.3(±1.87)	1.2(±1.8)
Group 2	(0.9)	(0.1)	(0.7)
(P value)			
ANC (cells/mL)			
Mean ±SD			
Group 1	1556(±891.8)	737.4(±718.6)	129.0(±69.9)
Group 2	1617(±929.1)	481(±325.9)	157.5(±26.1)
(P value)	(0.8)	(0.2)	(0.2)

Abbreviations: SD standard deviation; AA: Aplastic anemia; NSAA: non severe AA; SAA: severe AA; VSAA: very severe AA.

Hb: Hemoglobin; TLC: total leukocyte count; ANC: absolute neutrophil count

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Table 3: Comparison of VSAA+SAA cases with NSAA cases in Group 1 and 2

	AA WITHOUT PNH CLONE(Gp 1)n=43			AA WITH PNH CLONE(Gp 2) n=29		
	VSAA+SAA	**NSAA**	**P Value**	**VSAA+SAA**	**NSAA**	**P Value**
Hb (g/dl) mean	6.4	6.0	0.6	6.6	7.1	0.5
TLC ($\times 10^9$/L) mean	2.2	3.1	**0.05**	2.4	3.1	0.07
Platelet ($\times 10^9$/L) mean	17.7	69.3	**0.05**	16.2	65.5	**0.03**
ANC (×cells/L) mean	456.6	1556.1	**0.0001**	394.7	1617.9	**0.0002**
Reticulocyte (%) mean	0.7	2.3	**0.002**	1.3	2.3	0.1

Abbreviations: SD standard deviation; AA: Aplastic anemia; NSAA: non severe AA; SAA: severe AA; VSAA: very severe AA.

Hb: Hemoglobin; TLC: total leukocyte count; ANC: absolute neutrophil count

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Table 4: Comparison of VSAA+SAA cases within Groups 1 and 2

	AA WITHOUT PNH CLONE(Gp 1)n=26	**AA WITH PNH CLONE(Gp 2) n=15**	P Value
Hb (g/dl) mean	6.4	6.6	0.8
TLC ($\times 10^9$/L) mean	2.2	2.4	0.5
Platelet ($\times 10^9$/L) mean	17.2	16.2	0.5
ANC (cells/ml) mean	456.6	394.7	0.6
Reticulocyte (%) mean	0.7	1.3	0.2

Abbreviations: SD standard deviation; AA: Aplastic anemia; NSAA: non severe AA; SAA: severe AA; VSAA: very severe AA.

Hb: Hemoglobin; TLC: total leukocyte count; ANC: absolute neutrophil count

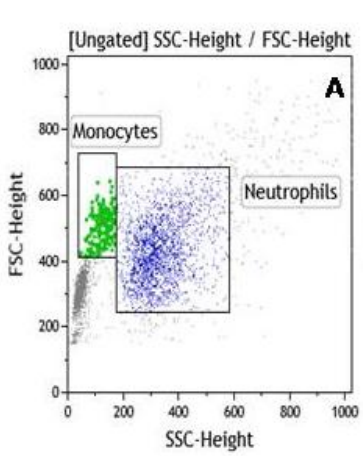

Figure 1A

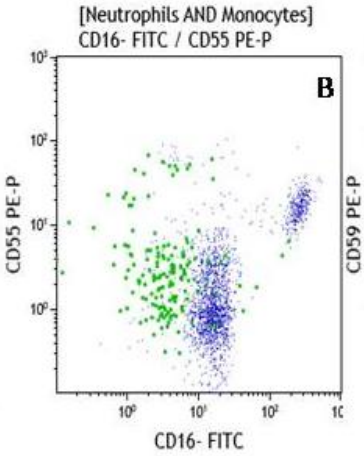

Figure 1B

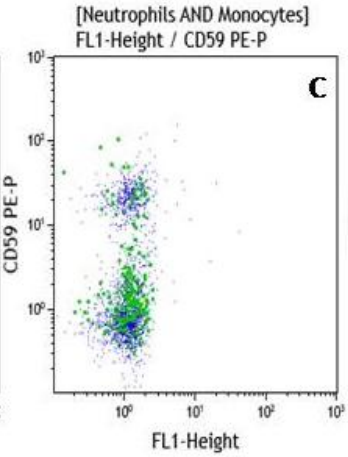

Figure 1C

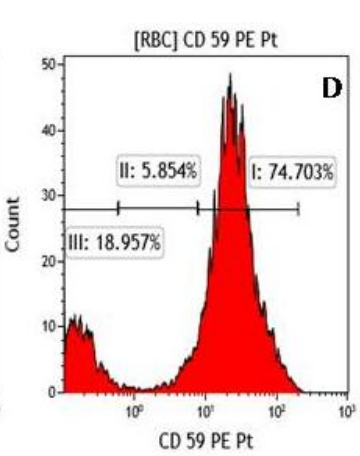

Figure 1D

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