Paroxysmal Nocturnal Hemoglobinuria Associated with *in vitro* Inhibition of Erythropoiesis by Bone Marrow T Lymphocytes

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Abstract

Background: The etiology of bone marrow failure, a prominent feature of paroxysmal nocturnal hemoglobulinuria, is presently unknown.

Objectives: To evaluate the possible influence of cellular immune mechanisms in the bone marrow failure of PNH.

Methods: We studied marrow erythroid colony formation in a patient with paroxysmal nocturnal hemoglobinuria without hypoplastic/aplastic marrow complications

Results: *In vitro* assays revealed a pronounced inhibition of primitive erythroid (BFU-E) progenitor cell growth by marrow T lymphocytes. Removal of T cells prior to culture resulted in a 4.5-fold enhancement of BFU-E numbers. Reevaluation of *in vitro* erythropoiesis during steroid administration indicated a persistent, albeit less prominent, T cell inhibitory effect.

Conclusion: Our findings provide the first direct evidence for a cellular immune inhibitory phenomenon accompanying PNH.

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This manuscript is dedicated to Dr. Rivka Sharon, Head of the Hematology Outpatient Clinic, who passed away at the time this investigation was performed.

Paroxysmal nocturnal hemoglobinuria is a syndrome characterized by intravascular hemolysis, venous thrombosis and bone marrow failure [1]. PNH often results in severe pancytopenia, either frank aplastic anemia or, rarely, myelodysplastic syndrome [2]. The PNH abnormality results from a somatic mutation/deletion of the X-linked gene PIG-A, encoding an enzyme component required for an early step in glycosylphosphatidylinositol synthesis [3,4]. The abnormality occurs in all blood cell types, suggesting its origin from a primitive hemopoietic progenitor.

The relationship between GPI deficiency and bone marrow failure in PNH remains unclear. It has been hypothesized that conditions damaging normal hemopoietic stem cells (i.e., immune suppression) may favor PNH stem cell expansion [5]. Although colony formation by hemopoietic progenitors in PNH is impaired [6–8], direct experimental evidence for immune suppression of hemopoiesis has not yet been provided. Indirect evidence for immune suppression comes from clinical studies demonstrating a favorable response of selected PNH patients with a hypoplastic/aplastic marrow to anti-thymocyte globulin [9] or cyclosporin A [10]. ATG or CSA did not eliminate the abnormal PNH clone, suggesting a specific effect on inhibitory immunocompetent cells.

We studied erythroid colony formation in bone marrow cultures of a patient presenting with unexplained hemolytic anemia. Our *in vitro* studies revealed a pronounced inhibition of primitive erythroid progenitor (BFU-E) cell growth by bone marrow T lymphocytes. PNH was subsequently diagnosed by flow cytometry in this patient. Our findings demonstrate, for the first time, the existence of a cellular immune inhibitory phenomenon accompanying PNH, even in the absence of aplastic/hypoplastic marrow complications.

Materials and Methods

A 21-year-old woman in the second trimester of pregnancy was referred to the hematology outpatient clinic in 1994 because of thrombocytopenia and normochromic refractory anemia. After delivery her platelet counts slowly returned to normal levels. However, a continuous decline in hemoglobin occurred. Bone marrow aspirate and biopsy showed hypercellularity, erythroid hyperplasia (53%) with megaloblastic features, without fibrosis.

Indirect bilirubin and low haptoglobin levels indicated hemolysis, but direct Coombs' test was negative. Since hemolysis alone could not explain the degree of anemia (Hb 7.7 g/dl, corrected reticulocytes 1.8%), these findings prompted an analysis of immune suppression in colony

PNH = paroxysmal nocturnal hemoglobinuria
GPI = glycosylphosphatidylinositol

ATG = anti-thymocyte globulin
CSA = cyclosporin A

assays of bone marrow erythroid progenitors. Subsequent to the findings the patient was given steroids, with no beneficial effect. Several months later, the severity of hemolysis increased and the patient required blood transfusions. Reevaluation of anemia revealed PNH, as detected by anti-CD59 and CD16 antibodies [11]. The patient began to take danazol, with reduced blood transfusion requirement and stable hemoglobin (7–8 g/dl). ATG or bone marrow transplantation from an HLA-compatible sister is being considered.

Preparation of marrow cells

Marrow aspirates from the patient and an age-matched healthy donor undergoing open heart surgery were obtained in heparinized syringes following informed consent. Cells were diluted threefold in Hanks' balanced salts solution and subjected to Ficoll-Hypaque gradient centrifugation. The buoyant mononuclear cells (<1.077 g/cm^3) were collected, washed three times in HBSS5 and resuspended for cell counting.

Removal of T cells

T cells were depleted from marrow mononuclear cells by rosetting separation with aminoethylisothioronium bromide-treated sheep red blood cells [12]. The T cell frequency following depletion was 0–0.5%.

Colony assay for BFU-E

Marrow mononuclear cells were cultured at 1x10^5 cells/ml modified Dulbecco's medium supplemented with 10% heat inactivated fetal calf serum (Beit HaEmek Biological Industries, Israel), 10 mg/ml bovine serum albumin (Sigma, Israel), 4x10^{-6} M iron-saturated transferrin (Sigma), 1x10^{-8} M sodium selenite (Sigma), 10^{-4} M 2-mercaptoethanol (Sigma), 1 g/L nucleosides (Sigma), and 1.5x10^{-5} M each of linoleic acid and cholesterol (Sigma), using 0.9% methylcellulose (Methocel A4M, premium grade, Dow Chemical Co., USA) as a viscous agent. Cultures (performed in triplicates) were incubated at 37°C in a fully humidified atmosphere of 7.5% CO_2 in air. For BFU-E growth, cultures were supplemented with 20 ng/ml recombinant human interleukin-3 (Genetics Institute, Cambridge, MA, USA) and 2 U/ml recombinant human erythropoietin (Boehringer Mannnheim GmbH, Germany). Colonies were enumerated following 14 days of culture.

Immunofluorescence staining and flow cytometry

For red blood cell analysis, fluorescein-isothiocyanate-conjugated anti-CD59 antibodies (Caltag, San Francisco, CA, USA) (1:300 dilution in phosphate-buffered saline) were added to 10 μl of whole blood drawn in EDTA. Analysis of granulocytes and T lymphocytes was performed following erythrocyte lysis. Granulocytes were labeled with phycoerythrin-conjugated anti-CD16 antibodies (IQ Immunoquality, Groningen, The Netherlands). For T lymphocytes, two-color cytometry was employed. Cells were first incubated with anti-CD3 antibodies conjugated to PE/Cyan 5 tandem (CyQ; IQ Immunoquality), washed three times and incubated again with FITC-conjugated anti-CD59 antibodies. Fluorescence was measured in a Coulter EPICS II flow cytometer.

HBSS = Hanks' balanced salts solution
PE = phycoerythrin
FITC = fluorescein-isothiocyanate

Results and Discussion

The diagnosis of PNH in our patient demonstrated the existence of discrete populations of abnormal CD59-negative erythrocytes and CD16-negative granulocytes (data not shown). As typically observed for PNH [13,14], only a small proportion (17%) of CD59-negative T lymphocytes could be detected in the same peripheral blood sample.

In vitro colony assays of the patient's bone marrow prior to PNH diagnosis failed to indicate any effect of autologous serum on BFU-E growth [Table 1]. However, removal of marrow T cells prior to culture resulted in a marked enhancement (4.5-fold) of colony numbers and a noticeable improvement in colony size and hemoglobinization.

Reevaluation more than a year later of *in vitro* erythropoiesis following PNH diagnosis and during danazol administration indicated a persistent, although less prominent, T cell inhibitory effect (1.8-fold colony enhancement). The attenuation of T cell inhibition at this time point may reflect the effect of danazol treatment, by an unknown mechanism. Interestingly, at this time point the abnormal clone could be detected by fluorescence-activated cell sorter analysis with anti-CD59 antibodies in the patient's marrow, but not peripheral blood cells. The enhancement of BFU-E numbers following T cell depletion of control marrow cells [Table 1] is compatible with progenitor enrichment per se, resulting from the T cell frequency (22%) in the marrow samples evaluated.

The possible involvement of inhibitory immune mechanism(s) in PNH has mainly been linked to an association between this disorder and aplastic anemia, both of which can occur simultaneously or evolve from each other [2,5,7]. Our patient currently has no signs of marrow hypoplasia or aplasia and thus may not meet the criteria for response to

Table 1. Effect of autologous serum and T cells on BFU-E growth in marrow cell cultures from the PNH patient

Treatment of marrow cells	BFU-E per 1x10^5 cells		
	4.2.97 Patient	4.6.98 Patient	Controls (n=5)
None	78±8	124±16	161±18
Autologous serum*	76±5	ND	168±22
Pooled AB serum	75±6	ND	163±16
T cell depletion	353±36 (x4.5)**	223±19 (x1.8)	209±22 (x1.3)
T cell frequency	10%	8%	22±3%

Patient results are expressed as mean ± SD of triplicate cultures.
* Cultures were supplemented with 10% autologous or pooled AB serum.
** BFU-E enrichment (-fold)

immunosuppressive therapy [9,10]. Nevertheless, our observation of marrow T cell inhibition not only warrants a trial with ATG or CSA in this patient, but also advocates *in vitro* cultures for screening inhibitory immune mechanism(s) in this complex and heterogenous disorder.

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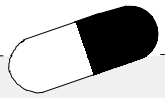
Life is not a matter of holding good cards. It's playing a poor hand well.

Robert Louis Stevenson

Capsule



Dietary fat and breast cancer

High intakes of fat and specific fatty acids, including total, animal, saturated, polyunsaturated and trans-unsaturated fats, have been postulated to increase breast cancer risk. In order to determine whether intakes of fat and fatty acids are associated with breast cancer, a cohort study was conducted in the United States beginning in 1976. The participants in this study (the Nurses' Health Study) comprised 88,795 women free of cancer in 1980 and followed for 14 years.

The results showed that 2,956 women were diagnosed as having breast cancer. Compared with women obtaining 30.1%–35% of energy from fat, women consuming 20% or less had a multivariate relative risk of breast cancer. In multivariate models, the RR for a 5%-of-energy increase was 0.97 for total fat, 0.98 for animal fat, 0.97 for vegetable fat, 0.94 for saturated fat, 0.91 for polyunsaturated fat, and 0.94 for monounsaturated fat. For a 1% increase in energy from trans-unsaturated fat and a 0.1% increase in energy from omega-3 fat from fish, the values were 0.92 and 1.09 respectively. In a model including fat, protein, and energy, the RR for a 5% increase in total fat, which can be interpreted as the risk of substituting this amount of fat for an equal amount of energy from carbohydrate, was 0.96. In similar models, no significant association of risk was evident with any major types of fat.

The authors conclude that there was no evidence that lower intake of total fat or specific major types of fat was associated with a decreased risk of breast cancer.

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