รายงานฉบับสมบูรณ์ยื่นต่อสำนักงานกองทุนสนับสนุนการวิจัย

Complete (Final) Report Submitted to the Thailand Research Fund

โครงการ: การศึกษาพยาธิกำเนิดของโรค Paroxysmal Nocturnal Hemoglobinuria
(BRG 02/2541)

Project Title: Studies of Pathogenesis of Paroxysmal Nocturnal Hemoglobinuria

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กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว) ที่สนับสนุนการวิจัยโครงการนี้ ผ่านทางทุนวิจัยเพื่อสร้างองค์ความรู้ใหม่ รหัสทุน BRG 02/2541 ตลอดจนคำแนะนำ (ของผู้ทรงคุณวุฒิ) ที่เป็นประโยชน์ต่อการวิจัยในโครงการ และความเอื้อเฟื้อที่อดทนรอความสำเร็จของโครงการที่ล่าช้ากว่ากำหนด เนื่องจากเหตุความจำเป็นบางประการ

ขอขอบคุณผู้ป่วยโรค PNH และโรคไขกระดูกฝ่อ (aplastic anemia) ทุกท่านที่ให้ความร่วมมือเข้าร่วมในโครงการนี้

ขอขอบคุณแพทย์และเจ้าหน้าที่ของสาขาวิชาโลหิตวิทยา ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล ทุกท่าน โดยเฉพาะอาจารย์และแพทย์ประจำบ้านที่ทุ่มเทกำลังกาย กำลังใจ และกำลังสมอง ในการดูแลรักษาผู้ป่วยเป็นอย่างดียิ่ง

บทคัดย่อ

Paroxysmal nocturnal hemoglobinuria (PNH) เป็นโรคของเซลล์ต้นกำเนิดเม็ดเลือด (hematopoietic stem cell disorder) ชนิด clonal มีลักษณะทางคลินิกที่สำคัญ คือ ภาวะเม็ดเลือดแดงแตกในหลอดเลือดชนิดเรื้อรัง (chronic intravascular hemolytic anemia) ภาวะสร้างเม็ดเลือดได้น้อย (deficient hematopoiesis) และภาวะการเกิดลิ่มเลือด (thrombosis) ได้ง่าย โรคนี้เกิดจากการกลายพันธุ์ (mutation) ชนิด somatic ของยีนที่อยู่บนโครโมโซม X ได้แก่ยีน PIG-A (phosphatidylinositol glycan class A) ในเซลล์ต้นกำเนิดเม็ดเลือด ทำให้เม็ดเลือดทั้งหลายขาดความสามารถในการสังเคราะห์ glycosylphosphatidylinositol (GPI) anchor ซึ่งส่งผลให้โปรตีนบางชนิด (GPI-linked proteins) ไม่สามารถแสดงออกที่ผนังเซลล์ได้ ในบรรดาโปรตีนเหล่านี้รวมถึงโปรตีนที่คอยควบคุมการกระตุ้นระบบคอมพลีเม้นต์ (complement) ได้แก่ CD59 และ CD55 ทำให้เม็ดเลือดแดงของผู้ป่วย PNH มีความไวต่อคอมพลีเม้นต์ และแตกสลายได้ง่ายในกระแสเลือด พบ PNH ในประเทศไทยมากกว่าประเทศตะวันตก ซึ่งเชื่อว่าเป็นผลจากการที่โรคนี้มีความสัมพันธ์กับโรคไขกระดูกฝ่อ (aplastic anemia) โครงการนี้ได้ศึกษาพยาธิกำเนิดของโรค PNH ใน 2 ประเด็น คือ 1) ศึกษาต้นกำเนิดและความสำคัญของเม็ดเลือด PNH (ความสัมพันธ์ระหว่าง genotype กับ phenotype) และ 2) กลไกการขยายตัวของ PNH clone ในประเด็นแรกทำโดยการตรวจหา PNH cell โดย flow cytometry และการศึกษาการกลายพันธุ์ของยีน PIG-A โดยการวิเคราะห์ heteroduplex และการตรวจหาลำดับนิวคลีโอไทด์ (nucleotide sequencing) พบว่าในผู้ป่วย PNH สัดส่วนของเม็ดเลือดแดง PNH (GPI-AP negative granulocytes) มีมากกว่าเม็ดเลือดแดง PNH (GPI-AP negative erythrocytes) และเป็นดัชนีแสดงขนาดของ PNH clone ได้ดีกว่า ชนิดของกลายพันธุ์ของยีน PIG-A ที่พบบ่อยคือ การขาดหายหรือเพิ่มขึ้นของ nucleotide จำนวนน้อยๆ (small deletion/insertion) และการแทนที่ nucleotide (base substitution) ทำให้ยีนนี้ไม่สามารถทำงานได้ เกิดเม็ดเลือดชนิด PNH III พบว่าความรุนแรงของโรค (โลหิตจาง) แสดงโดยขนาดของสัดส่วนของ GPI-AP negative granulocytes แต่มิใช่โดยชนิดของการกลายพันธุ์ของยีน GPI-A สำหรับการศึกษากลไกการขยายตัวของ PNH clone ทำโดยการตรวจสภาวะของการสร้างเม็ดเลือดในผู้ป่วย PNH (โดยการนับ CD34+ และการเพาะเลี้ยงเซลล์ต้นกำเนิดเม็ดเลือด) และการติดตามการเกิด PNH cells (clone) ในผู้ป่วยไขกระดูกฝ่อ (32 ราย) พบว่าผู้ป่วย PNH มีการสร้างเม็ดเลือดลดลง ซึ่งแสดงโดยจำนวน CD34+, BFU-E, และ CFU-GM ลดลง และพบว่า CD8+ ของผู้ป่วยเองทำให้ CFU-GM ลดลงไปอีก นอกจากนี้พบว่า CD59+ granulocytes ของผู้ป่วยมีการตายแบบ apoptosis มากกว่า CD59- granulocytes ประมาณ 70% ของผู้ป่วยไขกระดูกฝ่อมีการเกิดของ PNH clone ในระหว่างการศึกษา แต่การเกิด PNH clone ส่วนใหญ่มักจะเป็นอยู่ชั่วคราว และขณะที่เกิดนั้นผู้ป่วยมักจะมีจำนวนเม็ดเลือดขาวและเกร็ดเลือดต่ำกว่าขณะที่ไม่พบ PNH clone จากข้อมูลดังกล่าวแสดงว่า PNH clone ถูกเลือกโดยสภาวะหรือกลไกเดียวกับที่ทำให้เกิดภาวะไขกระดูกฝ่อหรือไขกระดูกทำงานล้มเหลว ซึ่งน่าจะเป็นกลไกออโตอิมมูน (autoimmune mechanism) หรือโดยขบวนการ immunoselection

คำหลัก: paroxysmal nocturnal hemoglobinuria (PNH), PIG-A, PNH clone

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder characterized clinically by chronic intravascular hemolytic anemia, deficient hematopoiesis and thrombotic tendency. It is originated from a somatic mutation of the X-linked phosphatidylinositol glycan class A (PIG-A) gene in a hematopoietic stem cell resulting in the inability to biosynthesize glycosylphosphatidylinositol (GPI) anchor with subsequent failure of GPI-anchored proteins (GPI-APs) to be expressed on blood cells. Deficiency of the GPI-linked complement regulatory proteins, CD59 and CD55, is responsible for the hypersusceptibility of erythrocytes to the lytic action of complements. It is believed the PNH is more common in East Asia including Thailand than in the western countries, possibly due to the geographical difference in the prevalence of aplastic which is closely related to PNH. Our proposed studies were conducted to elucidate two aspects of pathogenesis of the disorder; 1) the origin and significances of PNH-phenotype blood cells (genotype-phenotype study), and 2) mechanism of PNH clonal expansion. The first issue was explored by immunophenotypic study of blood cells by flow cytometry and detection of PIG-A mutations by heteroduplex analysis and nucleotide sequencing. We found that GPI-AP (CD59) negative granulocytes were more prominent than erythrocytes and could actually reflect the size of PNH clone. Somatic mutations of the PIG-A were found in all 37 patients studied, and were mostly small deletions/insertions and base substitutions causing frameshift or nonsense, leading to the common PNH III phenotype. Severity of the disease (anemia) was found to be determined by the proportion of GPI-negative granulocytes but not by the types of PIG-A mutations. The mechanism of PNH clonal expansion was explored by studying the condition of hematopoiesis in PNH patients (CD34+ enumeration and hematopoietic stem cell culture) and looking at nature of PNH clones in a cohort of 32 aplastic anemia patients. Approximately 70% of the aplastic anemia patients showed PNH clones (CD59- granulocytes) during the follow-up period. Most of the emerging clones were usually transient and appeared during the status of significant bone marrow failure as represented by more the prominent leucopenia and thrombocytopenia. By hematopoietic stem cell culture, we found that PNH is usually associated with a condition of deficient hematopoiesis as shown by the low CD34+ cells and also BFU-E and CFU-GM. In addition autologous CD8+ cells seemed to depress the CFU-GM. Moreover the CD59+ granulocytes were more apoptotic than the CD59- counterpart. Our findings suggested that PNH is selected in the condition which is also responsible for bone marrow failure in aplastic anemia, i.e., the autoimmune mechanism or the immunoselection.

Key words: paroxysmal nocturnal hemoglobinuria (PNH), PIG-A, PNH clone

สารบัญ

บทนำ

Paroxymal nocturnal hemoglobinuria (PNH) is an acquired non-malignant, clonal hematopoietic stem cell disorder, clinically characterized by chronic intravascular hemolysis with paroxysms of hemoglobinuria, a tendency to form venous thrombosis, and features of bone marrow failure as well as a close association with aplastic anemia[1]. PNH occurs in all population around the world. It is believed that PNH is more common East Asia including Thailand[2], probably because of the higher incidence of aplastic anemia in these regions[3].

PNH has been shown to originate through a somatic mutation of the X linked, phosphatidylinositolglycan class A (PIG-A) gene in a hematopoietic stem cell (HSC), followed by a considerable expansion of this abnormal (PNH) clone. PIG-A encodes one of the subunits of N-acetylglucosamine phosphatidylinositol transferase, the enzyme required in the first step of the glycosylphophatidylinositol (GPI) anchor biosynthesis. Mutation of this gene results in severe loss of the enzyme function with deficiency of the GPI, preventing the presence of membrane proteins which require this anchor[4]. As an X-chromosome gene, a single "hit" of the PIG-A in a HSC can generate a PNH phenotype in all types of blood cells because males have only one X chromosome and females have one X chromosome inactivated through lyonization. Although more than ten enzymatic steps are involved in the biosynthesis of GPI anchor, no case of PNH has been shown to occur as a result of mutation in any gene other than PIG-A[5,6]. This can be explained by the fact that other genes involved in the GPI-anchor synthesis are autosomal, and theoretically both alleles would need to mutate somatically to produce a loss of function.

More than a dozen of GPI-anchored proteins (GPI-APs) are missing from PNH blood cells, producing a variety of consequences. The most clinically significant consequences are observed in the red cells from the absence of two proteins, CD55 and CD59, which are responsible for regulating complement activity. CD55 or decay accelerating factor (DAF) controls the early part of the complement activity by regulating the activity of the C3 and C5 convertases. CD 59 or membrane inhibitor of reactive lysis (MIRL) controls the terminal complement by inhibiting the incorporation of C9 onto the C5b-8 thus preventing the formation of membrane attack complex (MAC). The observation of hemolytic anemia in an individual with the inherited CD59 deficiency[7] but not in those with inherited CD 55 deficiency (Inab-phenotype) suggests that missing of CD59 rather than CD55 renders PNH red cells vulnerable to complement-mediated lysis leading to intravascular hemolysis and its consequences in PNH.

As well as other clonal disorders, it is apparent that the clinical characteristics of PNH, especially intravascular hemolysis and thrombosis, occur in the presence of a large population of blood cells deficient in GPI-APs (a large PNH clone). It is a puzzle how the PIG-A mutated HSC clone expands under its non-malignant background. Mutation of PIG-A with the consequent deficiency of GPI-anchored molecules does

not confer any absolute growth advantage to the mutant cells and therefore, not explain the expansion of the PNH clone[8]. Clinical data particularly the remarkably close association between PNH and aplastic anemia suggest that the conditions of bone marrow failure selectively favor the expansion of PNH clone(s).

The main purposes of the project are to studies:

1) The relationships between PIG-A mutations, expression of the GPI-anchored proteins (CD59 and CD55), and clinical severity of PNH

2) The association of PNH and aplastic anemia by looking at the occurrence of PNH clones by immunophenotypic study of expression of the GPI-anchored proteins and PIG-A mutations in patients with aplastic anemia

3) Mechanism of PNH) clonal dominance by comparing the *in vitro* growth of CD59+ and CD59- hematopoietic stem cells (HSC) and the roles of co-culture with mononuclear cells.

วิธีการทดลอง

Patients

A total of 34 patients with PNH (24 males, 10 females) and a cohort of 40 patients with aplastic anemia (15 males, 17 females) followed at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, were included in the project. The diagnosis of PNH was based on clinical hemolytic anaemia and positive acidified serum lysis (Ham's) test. The diagnosis of aplastic anemia was based on the finding of peripheral blood pancytopenia and hypocellular marrow. All patients provided informed consent according to the Faculty's regulation before blood samplings. For the study of occurrence of PNH clones in aplastic anemia, the cohort period was 12-48 months (median, 20 months) with a 4 month interval between each investigation. The median number of immunophenotypic analyses was 4 per patient (range 3-17).

Immunuphenotypic detection of PNH clones

Expression of CD55 and CD59 on erythrocytes and granulocytes was analyzed by flow cytometry according to the method described previously[9]. Briefly, granulocytes were purified from heparinized venous blood by sedimentation through high molecular weight dextran and gradient centrifugation through Ficoll-Paque. EDTA blood was used for analysis of the erythrocytes. The PB samples were also collected into EDTA for immunophenotypic analysis and enumeration of reticulocytes by fluorescence-activated cell sorter (FACS) and for blood cell counts which were performed using an NE-1500 hematology analyzer (Sysmex, TOA Medical Electronics Co. Ltd., Kobe, Japan). The cells (granulocytes, erythrocytes and reticulocytes) were incubated with biotinylated monoclonal antibodies to DAF and to

CD59 (Mabs IA10 and 5H8, respectively, a gift from Dr M Tomita, Showa University, Tokyo) and then labeled with phycoerythrin (PE)-conjugated streptavidin (Biomeda, Foster City, CA). Mouse IgG1 and IgG2a were used as isotypic nonspeciÆc background staining controls. The cells were then analyzed in a FACScan (Becton Dickinson, San Jose, CA) using Lysys II and/or CellQuest software. For analysis of reticulocytes, DAF and CD59 staining was followed by 1-h incubation with Thiazole orange (TO, Becton Dickinson, San Jose, CA) with nonspecific background staining assessed by using phosphate-buffered saline instead of TO. The cells were analyzed in a FACScan using CellQuest software. Results of FACS analyses were reported as patterns according to the presence of blood cell populations with normal, intermediate, or complete deficient DAF or CD59 expression (PNH I, PNH II, and PNH III, respectively). The size of each population was also recorded as a percentage or proportion for each cell type. Based on the mean relative intensity of DAF or CD59-fluorescence, cells were distinguished into three discrete populations. Those with intensity that lay within the normal range (defined as mean±2SD) calculated from normal healthy controls (n=13) were of PNH I phenotype. Cells with intensity which lay within the DAF or CD59-negative area in the flow cytometric profile, defined as the area containing 99.8% of the negative control cells, were of PNH III phenotype. PNH II cells were found in between negative and normally positive areas.

Extraction of DNA and RNA from granulocytes

Granulocytes were isolated as described above. The purity of the isolated granulocytes was confirmed by flow cytometry. DNA and RNA were extracted from granulocytes by using Trizol reagent (Invitrogen, CA, USA), according to the manufacturer's instruction.

Polymerase chain reaction (PCR)

First strand cDNA synthesis was done by reverse transcription of total RNA using primer B13. The coding region of PIG-A was then amplified with proofreading polymerase Pfu using primers 11 (5'-ACCAGAGCTCGGTTGCTCTAAGAACTGATGTC) and 12 (5'-ACCAGGTACCTCTTACAATCTAGGCTTCCTTC). PCR products were separated by electrophoresis on 1 % agarose gel. The largest band (1,500 base pairs) which contained the entire coding region was purified from gel using QIAEX II gel extraction kit (Qiagen, Hilden, Germany) and used for PCR amplification or subcloned into pEB vector for transfection. The β-actin gene was amplified in parallel from the same amount of RNA as a control.

For PCR, DNA (100 ng) was amplified in reaction as described in previous reports[5, 10]. The PCR products were analyzed by electrophoresis on 2% agarose gel with ethidium bromide staining.

Heteroduplex analysis (HA) and DNA sequencing

The coding region of PIG-A was amplified from RT-PCR products of specific primers for PIG-A gene in six overlapping fragments, as described previously[5,10]. For heteroduplex formation, PCR products from the affected granulocytes were analyzed without or with mixing with corresponding PCR products from normal cDNA to obtain an approximately 1:1 ratio of the mutant and normal products. Complementary and genomic DNA of the regions which showed heteroduplex were prepared and subcloned into pGEM-T Easy (Promega, Madison, WI, USA) for sequencing. Several clones were sequenced in both directions using dye-terminator kit on an automatic sequencer ABI Prism model 310 (PE Applied Biosystems, Foster City, CA, USA).

Determination of functional activity of PIG-A cDNA

To determine PIG-A activity, we amplified PIG-A cDNA from patients and subcloned into pEB, an EBV-based expression vector. Twenty micrograms of DNA was then transfected into a PIG-A deficient mutant human B-lymphoblastoid cell line, JY-5 by electroporation at 250 V, 960 μF in 0.8 ml of HeBS buffer[11]. Two days later, the transfected cells were stained for CD55 and CD59 to assess the complementation of the PIG-A deficient phenotype of the mutant. Non-functional clones were then subjected to direct nucleotide sequencing to determine mutation.

Immunuphenotypic study of CD34+ cells

The CD34+ population was determined on CD45+ lymphocytes in the peripheral blood using MoAbs against CD45 and CD34 conjugated with fluorescein isothiocyanate and phycoerythrin (both from Becton Dickinson, San Jose, CA, USA), respectively [20]. Briefly, peripheral blood containing $1x10^6$ nucleated cells (volume not more than 50 μL) was incubated with the MoAb mixtures at 4°C for 30 minutes. After incubation, the erythrocytes were lysed by fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) at 4°C for 10 minutes. The remaining cells were washed twice with cold phosphate-buffered saline (PBS) containing 0.1% sodium azide and finally fixed with 0.5 mL of 0.5% paraformaldehyde in PBS for subsequent flow cytometric analysis using CellQuest software (Becton Dickinson).

Hematopoietic progenitor cell culture

The hematopoietic progenitor cell culture was done by the methylcellulose culture system, as previously described[12]. Briefly, mononuclear cells (MNCs) were separated from heparinized blood after centrifugation over Ficoll-Hypaque (Nycomed, Oslo, Norway). Cells were washed and resuspended in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, New York, NY, USA), and $5x10^5$ cells were cultured in 1 mL IMDM in the presence of 30% fetal bovine serum (Seromed, Berlin, Germany), 1% bovine serum albumin (Terry Fox Laboratories,Vancouver, Canada), 10–4 nmol α-thioglycerol (Sigma, St. Louis, MO, USA), and 0.8% methylcellulose (Sigma).

Cultures of erythroid burst-forming unit (BFU-E) and granulocyte-macrophage colonyforming unit (CFU-GM) myeloid colonies were performed by adding 1 U recombinant erythropoietin (Terry Fox) and 10% agar leukocyte-conditioned medium (Terry Fox), respectively. Each culture was duplicated in a 35-mm Petri dish (Nunc, Roskilde, Denmark) and incubated for 14 days in a fully humidified atmosphere of 95% air and 5% CO2 at 37°C. Colonies were enumerated on day 14 of the culture and expressed as numbers per $5x10^5$ MNCs.

In some experiments, the effects of CD8 or mononuclear cells on HSCs were studied. The CD8- cells were purified by using indirect immuno-magnetic activated cell sorter. MNCs were stained with 70 µl of anti-CD8 PE antibody to each 100 µl of resuspended MNCs (10^7/100 µl of PBS/EDTA) and incubated for 15 minutes at room temperature. Subsequently each 10^7 cells were incubated with 20 µl of goat anti-mouse IgG micro-beads for 15 minutes at 2^o to 8^oC. The CD8+ and CD8- fraction were fractionated by magnetic separation with LS column (Miltenyi Biotec, Earhart Avenue Auburn, CA, U.S.A.). Finally, 4 x 10^5 of CD8- cells and 1 x 10^5 of CD8+ were co-culture in methocult (Stem Cell Technologies, Vancouver, Canada) and plated into 35 mm culture dish in duplicate. As a control, 4 x 10^5 cells from CD8- cell fraction were also culture alone. Each culture was incubated for 14 days at 37^o C in 5% CO2 condition. Colonies consisting of at least 50 cells were enumerateded and characterized, according to their morphology, by an inverted microscope.

Flow cytometry analysis of apoptosis

1 x 10^6 PMNs were cultured alone as a control and co-cultured with 1 x 10^6 MNCs in 1 ml of RPMI-1640 supplemented with 1% L-glutamine, 10% FCS, 50 ug/ml streptomycin, and 50 IU/ml penicillin (Figure 1).

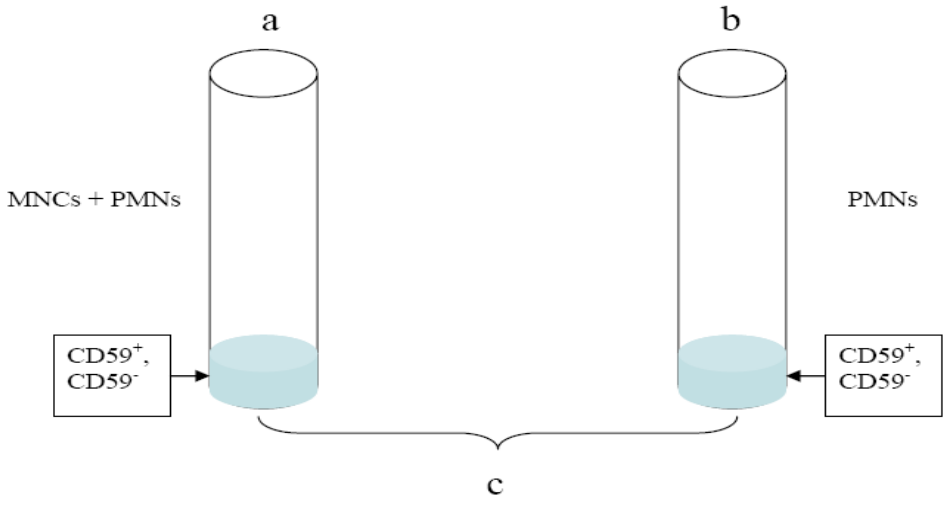
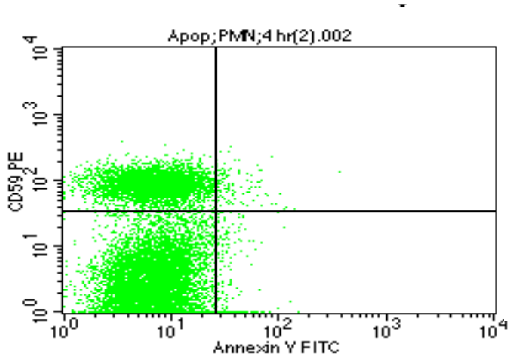


Figure 1 Analyses of apoptosis of CD59+ and CD59- granulocytes were performed in the same sample tube; (a) co-culture of PMN fraction with MNC fraction from the same individual, (b) culture of PMN alone, (c) study to compare between (a) and (b).

After culture for 0 and 4 hours, 0.5 ml of cell suspension were incubated with 50 µl of anti-CD59 biotin conjugated, 7 µl of anti-CD45 Per CP, 2 µl of annexin-V FITC, and 100 µl of binding buffer and incubated at 2o to 8oC for 15 minutes. Annexin-V binding in the CD59+ and CD59- cell populations was determined by flow cytometry based on three-color analysis. The percentages of apoptosis were expressed by the formula below.

$$\text{The percentages of apoptosis} = \left(\frac{\text{The number of apoptotic granulocytes (UR or LR)}}{\text{Total number of each granulocyte population}} \times 100 \right)$$



Quad	Events	
UL	4127	Total number of $CD59^+$ granulocytes
UR	114	
LL	8012	Total number of $CD59^-$ granulocytes
LR	225	

Statistical analysis

Except when indicated, data are expressed as means + standard deviation (SD). Differences between groups and parameters were evaluated by the Chi square and the unpaired t test. Pearson's correlation was used to determine the relationship between the proportions of the CD59 negative erythrocytes or granulocytes and hematological parameters. $P < 0.05$ was considered statistical significant.

ผลการทดลอง

Immunophenotypes of granulocytes and erythrocytes

As shown in Table I, immunophenotypic patterns of granulocytes and erythrocytes based on CD59 expression were determined for 26 and 27 patients, respectively. The most common pattern in both granulocytes and erythrocytes was PNH I+III (69% for granulocytes and 63% for erythrocytes), followed by PNH II+III for granulocytes (19%) and PNH I+II+III for erythrocytes (26%). 16 patients had different immunophenotypic features on granulocytes and erythrocytes. The most common discrepant pattern (seven patients) was PNH I+II+III erythrocytes but PNH I+III or II+III or III alone on granulocytes. The others included PNH I+III erythrocytes versus PNH II+III granulocytes (four patients). Five patients had granulocytes with only PNH III phenotypes; two of them had either PNH I+II+III or PNH II+III erythrocytes and the other three had PNH I+III erythrocytes.

Table I. Immunophenotypic patterns of granulocytes and erythrocytes based on CD59 expression.

Patterns of PNH cells	Granulocytes ($n = 26$ cases)	Erythrocytes ($n = 27$ cases)
I+III	18 (69%)	17 (63%)
II+III	5 (19%)	1 (4%)
I+II+III	—	7 (26%)
III alone	3 (12%)	—
Miscellaneous*	—	2 (7%)

* Included I+II and I alone.

By pairing the values of individual patients, granulocytes were significantly more affected than erythrocytes (two-tailed p=0.0001). Patterns of DAF and CD59 expression of reticulocytes were similar in affected reticulocytes to those of erythrocytes. The proportion of affected reticulocytes was somehow larger than the percentage of affected erythrocytes (Fig. 2; p<0.0001). The proportion of abnormal reticulocytes was, however, smaller than the proportion of affected granulocytes (p=0.021) (Figure 2).

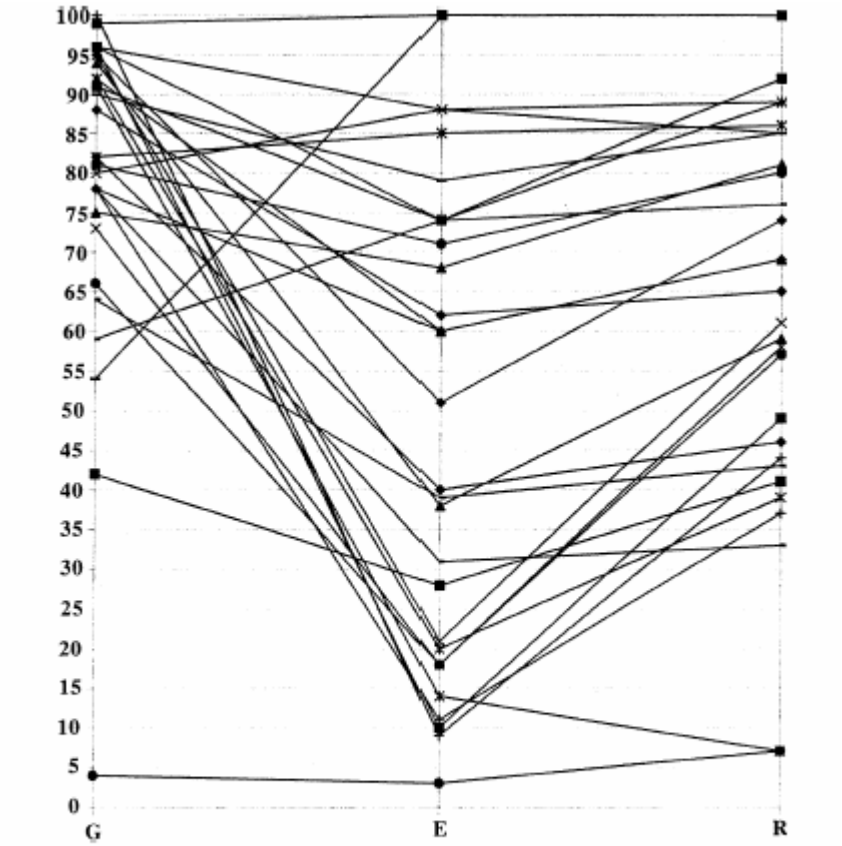


Fig. 2. Scattergram of percentage of abnormal (CD59 negative) granulocytes (G), erythrocytes (E), and reticulocytes (R) of each 29 PNH patients. The *Y*-axis represents percentage of CD59 negative cells.

Size of PNH clone and its significance

The size of the PNH clone (PNH II and/or PNH III) in the granulocyte lineage was estimated to be 6–98% (mean±SD 74·2±25·8). There was some, albeit not striking, negative correlation between size of PNH clone and mean basal hemoglobin concentration (r=-0·374; P=0·0476; Fig 3). However, there was a significant difference in size of PNH clone between patients with mild (64·9±29·5%) and severe (88·5±9·4%) anemia (P=0·01; Fig 4). There was no correlation between the size of the PNH clone with other clinical parameters including pre-existing aplastic anemia, granulocyte and platelet counts, Ham's test and responses to steroids.

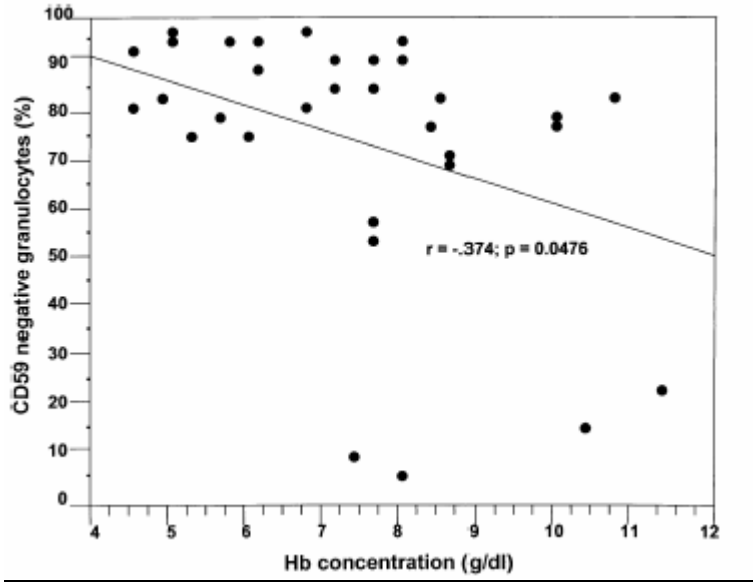


Fig 3 Scattergram to demonstrate negative correlation of basal hemoglobin concentration (horizontal axis) and CD59-negative granulocytes (vertical axis) (r = -0·374; P = 0·0476). Each dot indicates an individual patient.

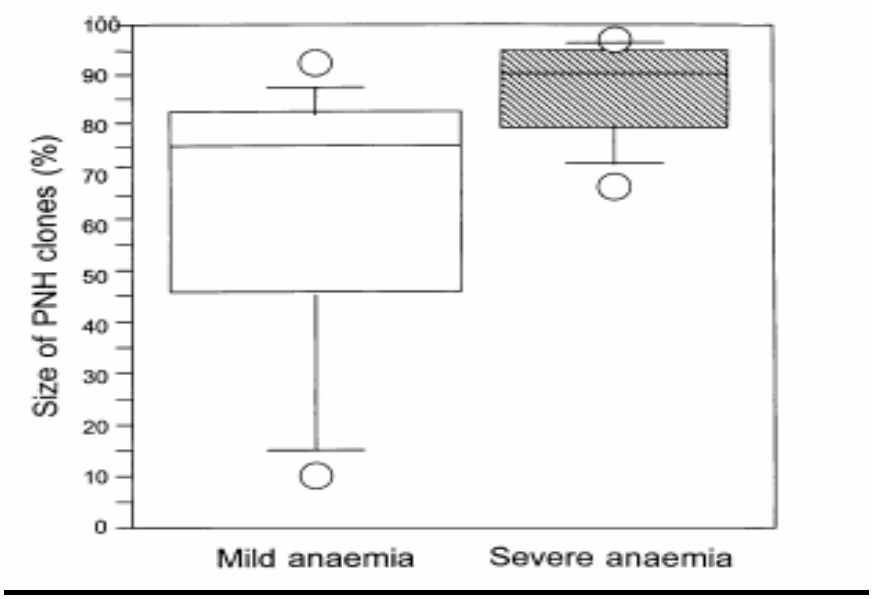


Fig 4 Comparison of size of PNH clones determined by percentages of CD59-negative granulocytes between patients with mild (mean±SD; 64·9±29·5) and severe (mean±SD; 88·5±9·4) anemia, P = 0·01. The results are shown as box-plot; medians, 25th–75th, 10th–90th percentile, as well as extreme values (open circles).

Somatic mutation of PIG-A

We determined 37 mutations in 34 patients; 14 of them (T1–T14) have been previously reported[5] (Table II). All of them were small mutations scattered throughout the coding regions with no striking 'hot spot'. The majority of the mutations were deletion (19) or insertion (five) mutations (together 65%). Among them one-base deletions were the most common mutations (14/37 or 38%). The largest deletional and insertional mutations found were 10-base deletion and 19-base insertion. The most common consequence of the mutations was a frameshift (27/37) that caused a premature stop codon. One of 10-base deletion mutations at codon 284–287 caused deletion of the entire exon 4 in mRNA. The one three-base deletion (TTC) caused a deletion of phenylalanine (Phe) at codon 151. Ten mutations (27%) were single base changes. Three of these point mutations changed one amino acid to another in the protein sequence (missense mutations) and two brought an immediate termination codon (nonsense mutations). There were two cases of G to A change in the 30 splice site of intron 5 that caused one base shift of the splice site, resulting in frameshift. Another base change, A to G, in the same splice site caused an abnormal splicing and low mRNA level. Two mutations in the 50 splice site of intron 4 and intron 5 caused deletions of the preceding exons from mRNA. Four patients had two mutations

detected at the same time. All of them were independent mutant clones. They included combination of 2 bp deletion and 19 bp insertion, two single base changes, 1 bp deletion and one base change, and 1 bp deletion and a change of 3 bp to 2 bp.

We compared 37 mutations found in Thai patients with those found in Japanese patients (37 mutations) and European/American patients (56 mutations) (Table III). There was no significant difference among the profiles of mutations in three groups. Single-base substitutions and one-base deletions were two major types of mutations, comprising 65–68%.

Table II. Somatic mutations of PIG-A.

Patient	Sex	CD59-deficient PMN (%)	Site/codon	Mutation	Consequence
T1	M	70	Codon 407	T deletion	Frameshift
T2	M	50	Codon 412	C deletion	Frameshift
T3	M	96	Codon 427	T deletion	Frameshift
T4	M	9	Codons 284–287	10-base deletion (TGCGTCTTTT)	Exon 4 deletion
T5	F	86	Codon 209	T deletion	Frameshift
T6	M	80	Codon 272	A deletion	Frameshift
T7	F	21	Codon 203	2-base deletion (GTG to G)	Frameshift
T8	M	69	Codon 248	C deletion	Frameshift
T9	M	82	Codon 151	3-base deletion (TTC)	Phe deletion
T10	M	55	5′ splice site	G to A, base change	Exon 5 deletion
T11	F	81	5′ splice site	G to A, base change	Exon 4 deletion
T12	M	95	Codons 193/194	5-base deletion (GTACT)	Frameshift
T13	M	70	Codon 135	T insertion (GCC to GTCC)	Frameshift
T14	M	6	Codon 299	T insertion (TTA to TTTA)	Frameshift
T15	M	84	Codon 128	A to G, base change	His to Arg
T16	M	70	Codons 227–233	19-base insertion (AACTATTGTTGTTGTCAGC)	Frameshift
			Codon 119	2-base deletion (CGG to C)	Frameshift
T17	M	90	3′ splice site of intron 5	G to A base change	Frameshift due to abnormal splicing
T18	M	98	Codon 169	A insertion	Frameshift
T19	F	92	Codon 211	T deletion	Frameshift
T20	M	80	Codon 263	G to A, base change (GGA to AGA)	Gly to Arg
			3′ splice site of intron 5	G to A, base change	Frameshift due to abnormal splicing
T21	M	98	Codon 83	C deletion (CTC to TC)	Frameshift
			Codons 367/368	AGT to TG	Frameshift
T22	M	92	Codon 323	A deletion	Frameshift
			Codon 77	C to T, base change	Stop
T23	M	94	Codon 212	C deletion	Frameshift
T24	M	82	Codon 239	G to T, base change	Gly to Val
T25	M	12	Codon 287	2-base insertion (TTG to TTTGG)	Frameshift
T26	F	77	Codon 164	C to A, base change	Stop
T27	F	96		Not found	
T28	F	77	Codons 418–422	11-base deletion/1-base insertion (TCACTGCGGCC to G)	Frameshift
T29	M	95	Codon 371	T deletion	Frameshift
T30	F	81	Codon 344	T deletion	Frameshift
T31	M	ND	Codon 49	2-base change (GT to AA)	Val to Leu
T32	F	90	Codon 132	T deletion	Frameshift
T33	F	92	Codon 65	C deletion	Frameshift
T34	M	78	3′ splice site of intron 5	A to G, base change	Abnormal splicing caused low mRNA

Table III. Comparison of somatic mutations of PIG-A found in patients with PNH from various geographical areas.

	Thailand	Japan	Europe/U.S.A.	Worldwide
Base substitutions	10 (27%)	16 (43%)	21 (38%)	47 (36%)
One-base deletions	14 (38%)	9 (24%)	17 (30%)	40 (31%)
More than one base deletions	5 (14%)	7 (19%)	4 (7%)	16 (12%)
One-base insertions	3 (8%)	3 (8%)	6 (11%)	12 (9%)
More than one base insertions	2 (5%)	1 (3%)	5 (9%)	8 (6%)
Others	3 (8%)	1 (3%)	3 (5%)	7 (5%)
Total	37 (100%)	37 (100%)	56 (100%)	130 (100%)

Relationship between PIG-A mutations and immunophenotypes (Table IV)

Mutations of the PIG-A gene leading to frameshift, exon deletion, and stop codon (nonsense) were responsible for the type III PNH phenotype. All three base-change mutations at the 30 splice site of intron 5 causing abnormal splicing were likely to be associated with type III phenotypes. Three missense mutations, i.e. A to G base change at codon 128 (amino acid change His to Arg), G to T at codon 239 (Gly to Val) and G to A at codon 263 (Gly to Arg), led to complete loss of function of PIG-A since they were associated with the only PNH III of both granulocytes and erythrocytes. One missense mutation of two-base change at codon 49 with amino acid change from Val to Leu was found in a patient with PNH I+II erythrocytes. This mutation is likely to be responsible for the PNH II phenotype.

We could not identify mutations responsible for PNH II in the majority of patients who had PNH II+III granulocytes or erythrocytes.

Two other base substitutions in PIG-A not relevant to CD59 deficiency

We found one more base substitution in patients T18 and T32. One, found in T32 was a G to C change that caused Arg to Thr change at codon 29. This female patient had a T deletion in codon 132. 90% of this patient's PMNs were CD59-negative and five of six cDNA clones derived from PMN had the T deletion. All these six cDNA had the base substitution. This indicated that the T deletion and the base substitution were present on the same allele. The amino acid change at codon 29 probably had no significant effect on CD59 expression, because yeast PIG-A homologue does not have the N-terminal region including codon 29 and because this Arg is not conserved in nematode of PIG-A. Whether this base substitution is constitutional or somatic is not yet clear.

Table IV. Relationships between consequences of PIG-A mutation and immunophenotypes.

Immunophenotype (no. of patients)	Consequences of PIG-A mutation					
	Frameshift	Missense	Exon deletion	Nonsense	Abnormal splicing	Amino acid deletion
Granulocytes						
(I)+III (21*)						
Single mutation (17)	9	2[a]	2	1	2	1[c]
Two mutations (3)	4	1[b]	—	—	1	—
II+III (5)						
Single mutation (4)	4	—	—	—	—	—
Two mutations (1)	1	—	—	1	—	—
Erythrocytes						
(I)+III (17*)						
Single mutation (14)	8	2[a]	2	1	—	1[c]
Two mutations (2)	2	1[b]	—	—	1	—
(I)+II+III (8)						
Single mutation (6)	4	—	—	—	2	—
Two mutations (2)	3	—	—	1	—	—
Miscellaneous (2)	1	1	—	—	—	—

* Included one patient with no mutation detected.

[a] A to G at codon 128; His → Arg; G to T at codon 239; Gly → Val. [b] G to A at codon 263; Gly → Arg. [c] Three-base (TTC) deletion caused Phe 151 deletion.

The association of PNH and aplastic anemia

1) Frequency, size and nature of the PNH clones in aplastic anemia patients

Among the total cohort 167 flow cytometric analyses in the 32 patients, 51 episodes (31%) of significant occurrences of CD59 negative granulocytes were observed in 22 patients (69%). CD59 negative erythrocytes were less apparent than the granulocytes, being detected in only 7 patients (22%). All of them also had concurrent CD59 negative granulocytes. Repeated appearances of PNH granulocytes and erythrocytes were found in 16 (50%) and 6 (19%) patients, respectively. At least 70 % of the emerging PNH granulocytic clones were transient in nature.

The size of the emerging PNH clones as measured by the proportion of CD59 negative cells varied, 1.0-97% (median, 4.3%) for the granulocytes and 1.04-16.8% (median, 4.8%) for the erythrocytes (Table V).

Based on the occurrence of PNH cells, we can divide our aplastic anemia patients into three groups.

Group 1 (n=10). Patients in this group did not show PNH granulocytes nor erythrocytes during follow-up period.

Group 2 (n=15). Transient appearances (up to four times) of PNH granulocytes were demonstrated in 15 patients. Three of them also had transient occurrences of PNH erythrocytes. Example of this pattern was shown in patients AP. 7 & 9 in Fig. 5.

Group 3 (n=7). There were seven patients who had persistent PNH granulocytic clones and four of them (AP. 8, 11, 12, and 32) also had persistent and increasing PNH erythrocytic clones with significant positive acidified serum test (>2% lysis). Two of the four patients subsequently developed hemolytic anemia (clinical PNH).

Table V Occurrence of PNH (CD59 negative) blood cells in aplastic anemia patients

	PNH granulocytes	PNH erythrocytes
No. of patients with:	22	7
- Transient occurrence	15	3
one episode	6	1
two or more episodes	9	2
- Persistent occurrence	7	4
Episodes of occurrence (total no. of studies = 167)	51 (31%)	21 (12.6%)
Size of PNH clone; median (range)	4.3 (1.0–97.0)	4.8 (1.04–16.8)

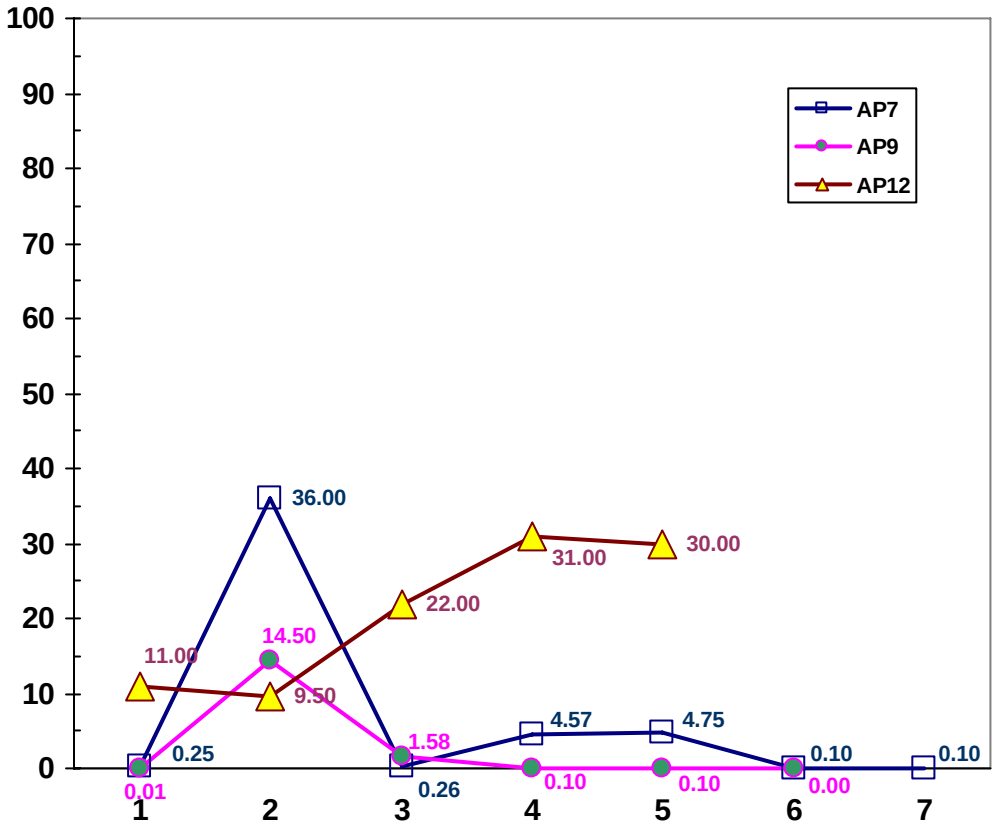


Figure 5 Size (%) of CD59 negative (PNH) granulocytes of representative patients AP7, AP9 and AP12 at different sequential times of flow cytometric studies. The number at each spot represents the actual percentage of the CD59 negative cells. Note the transient occurrences of the PNH clones in the patients AP7 and AP9 (more than one episodes), and the persistent and expanded clone in AP12.

2) Factors determining occurrences of the PNH clones (Table VI)

We looked for the clinical features and hematological parameters which determined the occurrences of the PNH clones represented by the CD59-negative granulocytes. There was no apparent change in the clinical characteristics during the presence of PNH clones. Only two of the four patients with persistent PNH clones had clinical features of hemolytic anemia i.e., increasing anemia, dark urine, at seven and twelve months after the first detections of GPI-negative granulocytes.

There was no direct correlation between the proportions of CD59-negative granulocytes and hematological data such as hematocrit/hemoglobin concentrations, white blood cell (WBC) and platelet counts. However, PNH clones were more frequently detected during episodes of lower white blood cell counts (3,203 + 1,133

vs 4,197 + 1,706/cu.mm.; $p < 0.001$) and lower platelet counts (24,419 + 20,262 *vs* 38,346 + 31,715/cu.mm; $p = 0.0121$), (Table 3). In addition, CD59-negative granulocytes were more frequently detected when patients' hemoglobin concentrations were < 12 g/dL than when > 12 g/dL (45 and 6 episodes out of 99 and 65 analyses, respectively; p<0.005) (Table VI).

Table VI Conditions associated with the occurrence of PNH granulocytes

PNH granulocytes	Episodes when Hb		WBC [b] (10^9/L)	Platelet count [c] (10^9/L)
	< 12 g/dl	≥ 12 g/dl		
Positive [a]	45	6	3.2 ± 1.1	24.4 ± 20.3
Negative	54	59	4.2 ± 1.7	38.3 + 31.7

a: 45/45 + 54 vs. 6/6 + 59 ; p < 0.005
b: p = 0.001
c: p = 0.012

3) Detection of PIG-A mutations in aplastic anemia

Mutations of PIG-A were successfully analyzed in the four patients (nos. 8, 11, 12, and 32) who had persistent PNH granulocytic as well as erythrocytic clones (Table VII). All of them showed normal pattern of RT-PCR products of 1,500 and 850 bps. HA located regions containing mutations in two patients. Sequencing of these pGEM-T subcloned regions revealed T deletion in codon 274 and G deletion in codon 146 of the patients' nos 11 and 32, respectively. Both mutations led to frame shift consequences.

Since an initial screening by HA could not detect area of mutation in patients nos. 8 and 12, we purified the coding regions of the PIG-A, and subcloned into pEB vector and assessed the functional activity as described above. We obtained several non-functional clones which were subjected to direct sequencing. All mutations were confirmed by re-amplification, subcloning and sequencing of several clones. Four different mutations were found in patient no.8 (Table VII), three of them resulted in changes of amino acids and one with a nonsense consequence. Two non-functional mutations of PIG-A were detected in patient no.12 (Table VII) one was a 14-base deletion with frame shift consequence, and the other was a base change with a stop codon.

Table VII PIG – A mutations in patients with aplastic anemia

Patients (AP)	CD59 negative* granulocytes (%)	Non-functional + transfected clone (%)	Mutation	Site codon	Consequence
8	50	3/9 (33)	G to T	183	Cys to Phe
			C to T	301	stop codon
			T to C	306	Leu to Pro
			C to T	331	Thr to Met
11	77	ND	T deletion	274	frame shift
12	28	2/10 (20)	G to A	287	stop codon
			14 base deletion	328 - 332	frame shift
32	62	ND	G deletion	146	frame shift

* % CD 59 negative granulocytes at the time of RNA/DNA extraction
+ Non functional / total transfected clones in pEB vector
ND = Not done

Hematopoietic Colony-Forming Cells

The hematopoietic progenitor cell culture demonstrated deficient hematopoiesis in PNH. Among the 13 patients studied, BFU-E and CFU-GM were markedly diminished compared with normal controls (n = 18) (BFU-E, 2.8 ± 1.2 versus 25.6 ± 6.2/5x10^5 MNCs, *P* = .0006; and CFU-GM, 1.2 ± 0.5 versus 13.3 ± 3.0/5x10^5 MNCs; *P* = .0006) (Figure 6A and B). Except for 1 patient (no. 13), the diminution of both CFU-GM and BFU-E were concordant with the number of CD34+ cells (Table VIII). The findings suggest that a low number of hematopoietic stem cells is one of the factors responsible for the diminished hematopoiesis in PNH. The condition of deficient hematopoiesis occurs irrespective of precedent AA or cellularity of the bone marrow (Table VIII).

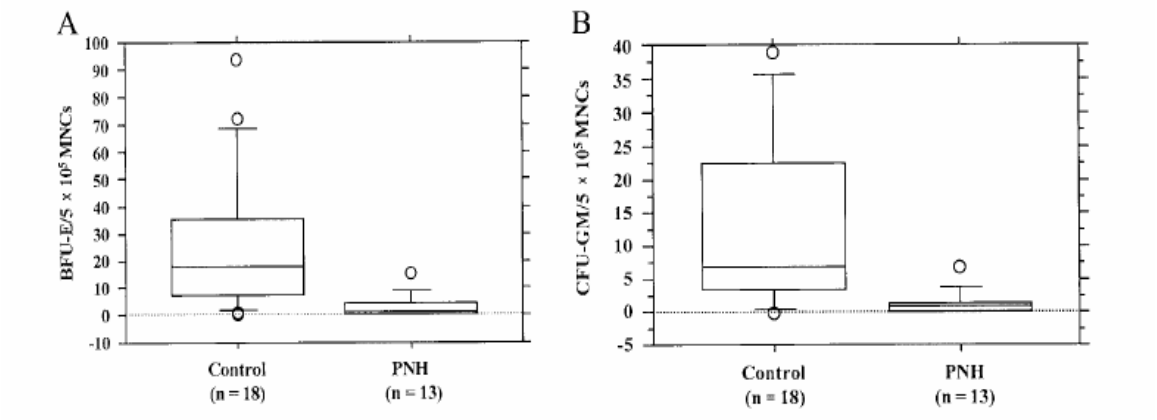


Fig 6 Colony-forming cells in peripheral blood of patients with paroxysmal nocturnal hemoglobinuria (PNH) compared with normal subjects. A, Erythroid burst-forming units (BFU-E). B, Granulocyte-macrophage colony-forming units (CFU-GM). The results are shown in box-plot. MNC indicates mononuclear cell.

Table VIII

CD34⁺ Cells, Erythroid Burst-Forming Units (BFU-E), and Granulocyte-Macrophage Colony-Forming Units (CFU-GM) in 15 Paroxysmal Nocturnal Hemoglobinuria Patients*

Patient	CD34⁺ Cells, ×10⁶/L	BFU-E/5 × 10⁵ MNCs	CFU-GM/5 × 10⁵ MNCs	History of Aplastic Anemia	Bone Marrow Status
1	0.62	ND	ND	No	ND
2	ND	1	1	No	ND
3	0.72	ND	ND	No	ND
4	1.75	1	0	No	ND
5	ND	3	7	No	ND
6	ND	0	0	No	ND
7	3.14	5	1	Yes	Hypocellular
8	0.49	0	0	Yes	ND
9	ND	1	0	Yes	Hypercellular
10	0.84	2	0	No	ND
11	ND	6	3	Yes	Hypercellular
12	ND	0	1	No	Hypocellular
13	10.88	0	0	No	Hypocellular
14	1.06	ND	2	Yes	Hypocellular
15	ND	15	1	Yes	Hypercellular

*MNC indicates mononuclear cell; ND, not done.

Hematopoietic colony-forming cells in the presence of CD8

In normal controls, the numbers of BFU-E and CFU-GM were not different when compared between cultures were performed with or without the CD8+ fraction (mean ± SEM; 220.30 ± 20.85 *vs* 234.60 ± 23.26, P = 0.18 for BFU-E and 15.70 ± 2.65 *vs* 16.90 ± 3.69, P = 0.59 for CFU-GM).

In contrast, the numbers of CFU-GM of PNH patients were lower in culture with the presence of CD8+ fraction than those in culture without CD8+ cells. (mean ± SEM; 1.50 ± 0.71 *vs* 3.10 ± 1.16, P = 0.04 for CFU-GM and 28.80 ± 8.96 *vs* 30.20 ± 10.06, P = 0.67 for BFU-E) (Fig 7).

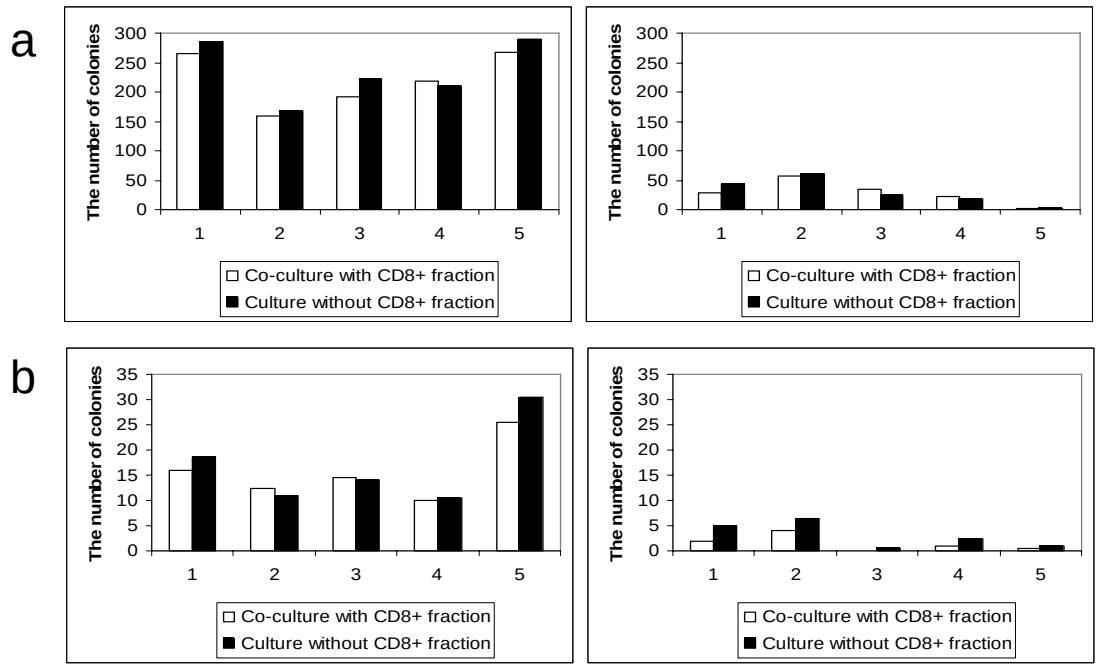


Fig 7. Hematopoietic cell culture with and without CD8+ T cells. (a) The number of BFU-E hemopoietic progenitor colonies of normal controls (left) and PNH patients (right) (b) The number of CFU-GM hemopoietic progenitor colonies of normal controls (left) and PNH patients (right).

Roles of apoptosis in selection of PNH cells

The percentages of apoptosis in each target population were shown in Table IX. After 0-or 4-hour in liquid growth culture system, CD59+ granulocytes (PMNs) from PNH patients exhibited more apoptosis than the CD59- counterparts (mean ± SEM; 2.12 ± 0.47 *vs* 1.20 ± 0.20, p=0.01 at 0 hour and 3.35 ± 0.67 *vs* 1.82 ± 0.26, p=0.03 at 4 hour respectively). When compared with normal controls, CD59+ granulocytes from PNH patients were more apoptotic than the CD59+ granulocytes from normal controls.
Co-culture of granulocytes with mononuclear cells for 4 hours rendered more difference in apoptosis between the CD59+ granulocytes (3.35 ± 0.66) and the CD59- cells (1.67 ± 0.29), p=0.005.

Table IX The percentages of apoptosis of PMNs from 33 normal controls and 15 PNH patients represented in mean± SEM (ND = not done)

	% of apoptosis of PMNs culture without MNCs				% of apoptosis of PMNs co-culture with MNCs			
Time of co-culture	0 hr		4 hr		0 hr		4 hr	
Cell population	CD59(+)	CD59(-)	CD59(+)	CD59(-)	CD59(+)	CD59(-)	CD59(+)	CD59(-)
Normal controls	0.74 ± 0.11	ND	3.24 ± 0.52	ND	0.68 ± 0.07	ND	2.10 ± 0.37	ND
PNH patients	2.12 ± 0.47	1.20 ± 0.20	3.35 ± 0.67	1.82 ± 0.26	1.78 ± 0.33	1.31 ± 0.27	3.35 ± 0.66	1.67 ± 0.29

บทวิจารณ์และสรุป

PIG-A mutation and genotype-phenotype relationship

In this study, data on the somatic mutations of PIG A are from a large homogenous group. Findings of two mutations in each of four patients support the fact that several different PNH clones may co-exist[13] and suggest that somatic mutation of PIG-A is not an uncommon phenomenon. The types of defects in the PIG-A gene among Thai PNH patients (Table III) were probably not different from the overall abnormalities in PIG-A gene so far reported[13,14]. The results suggest that there are no clear-cut differences in the mutations of PIG-A among different regions or ethnic groups.

Our study of somatic mutations of the PIG-A demonstrated that the majority of deletion/insertion mutations resulted in a frameshift consequence with premature stop codon. These PIG-A abnormalities are probably responsible for PNH III cells, since such defects would not result in a functional protein product. A phenylalanine in codon 151 is crucial for the function of PIG-A protein, since its deletion was responsible for the PNH III phenotype. The other consequences of PIG-A mutations responsible for PNH III cells included nonsense, abnormal splicing and exon deletion. Of the three missense mutations which were responsible for the complete loss of functions of the PIG-A, one, Gly to Arg at codon 263, was associated with a change of surface charge. There are no possible explanations for the two missense consequences, i.e. His to Arg at codon 128 and Gly to Val at codon 239. A missense mutation with an uncharged amino acid change of Val to Leu at codon 49 was probably responsible for PNH II erythrocytes in one patient whose immunophenotype of granulocytes was not determined. We have not seen any relationship between pattern of PIG-A defects and size of PNH clone, severity of anemia, preexisting aplastic anemia, and granulocyte and platelet counts.

Our study demonstrated some discrepancy in the immunophenotypic pattern between erythrocytes and granulocytes. Since the circulating life-span of the abnormal PNH erythrocytes is affected by complement-mediated hemolysis, it is possible that most of the complement sensitive cells were already hemolysed intravascularly at the time of study. Because PNH granulocytes have a normal life-span[15], their proportion probably more closely reflect the size of PNH stem cells. In our study the clinical significance of the PNH clone was determined according to the proportion of affected granulocytes. There was minimal direct negative correlation between the size of the PNH clone and hemoglobin concentration level. However, patients with severe anemia had a significantly higher proportion of affected granulocytes than those with mild anemia. The findings suggest that size of the abnormal clone is one of the factors which determines the severity of the disease, manifested as anemia. There are probably other factors responsible for severity of anemia such as hemopoietic activity , and environmental factors such as infections which exacerbate hemolysis. Our study did not show any relationship between immunophenotypes and other clinical features such as pre-existing aplastic anemia, neutrophil and platelet counts and Ham's test (data not shown).

The association of PNH and aplastic anemia

In our longitudinal study, 70% of the cohort aplastic anemia patients had significantly detectable PNH granulocytes during their clinical courses with a 31% frequency of total studies. Half of the patients had more than one episodes of occurrence of the PNH clones during follow up periods. Our study emphasized, as with the others[16,17], not only a high frequency of PIG-A mutations but also a high probability of expansion of PNH clones to a certain detectable levels. Interestingly, we found that most of the emerging PNH clones in these patients were transient in nature. This finding was different from a previous study[18] which demonstrated a persistent occurrence of PNH

cells in more than 50% of their patients. Immunosuppressive therapy may be responsible for the difference since nearly all of our patients received anabolic hormones, but not immunosuppressive agents such as anti-thymocyte globulin (ATG) or cyclosporine as a treatment.

Our study showed that the appearances of GPI-AP negative granulocytes were associated with the condition of bone marrow failure as demonstrated by more severe leucopenia and thrombocytopenia and also anemia (Table VI). Our finding is consistent with the proposed hypothesis[19] that PNH clones may survive pathologic conditions that lead to bone marrow failure in aplastic anemia as indicated by the degrees of cytopenias. As pancytopenia and hypocellular bone marrow in aplastic anemia are due to damage of the hematopoietic stem cells, most likely mediated by autoimmune process through the T-cell cytotoxic repertoire. GPI-deficient or PNH cells may escape immunological attack by the autoreactive cytotoxic T cells.

PNH hematopoiesis and the roles of CD8 and apoptosis

Our study demonstrated the defective hematopoiesis in PNH. Circulating CD34+ cells, which represent immature hematopoietic progenitor cells, were numerically decreased in PNH. The finding occurred in PNH patients with or without history of acquired AA, although it was more obvious in patients who had PNH following clinical AA. In addition, the low numbers of circulating CD34+ cells occurred irrespective of bone marrow cellularity. It is likely, therefore, that the diminished CD34+ cells were consistent in both the hemolytic and aplastic types of PNH, as observed by others[20].
In addition to the diminished CD34+ cells, the committed hematopoietic cells of erythroid (BFU-E) and myeloid (CFU-GM) series were also decreased compared with normal subjects. The results of our study extend previous observations by our group and others showing that the numbers of progenitor cells of both BFU-E and CFU-GM in the blood and bone marrow were numerically diminished in both aplastic and hemolytic types of PNH, although the patients with marrow hypocellularity had lower numbers of BFU-E and CFU-GM than did those with hypercellular marrow[21]. Our study indicates that there has been defective hematopoiesis in PNH irrespective of preexisting anemia or whether aplasia is still present in the bone marrow. It also supports the clinical observation that the presence of bone marrow failure may have a role in the pathogenesis of PNH.

With the co-culture studies, we found that in patients with PNH autologous CD8+ lymphocytes were shown to inhibit CFU-GM but not BFU-E formation. It is possible that CFU-GM may be more sensitive to cytotoxic damage by the autologous CD8+ lymphocytes than BFU-E. This finding also suggests that PNH clones are selected through autoimmune process which is also responsible for the condition of bone marrow failure. In addition, we found that CD59+ granulocytes from PNH patients were more apoptotic than their CD59- counterparts. The presence of autologous MNCs for 4 hours rendered more difference in apoptosis between CD59+ and CD59- granulocytes. Moreover, of CD59+ granulocytes from PNH patients were more

sensitive to apoptosis than the CD59+ granulocytes from healthy people. Taken together, our findings suggest that the expansion of the PNH clones could occur via autoimmune selection (partly through differential apoptosis) induced by autologous mononuclear cells in the fraction of the CD8+ lymphocytes, as GPI-deficient or PNH cells may escape immunological attack by the autoreactive cytotoxic T cells.

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Declaration

Parts of the message in this report also appeared in the publications as indicated in the output section and should not be otherwise distributed or re-publicized.

OUTPUT ที่ได้ (total 6)

(แนบอยู่ในภาคผนวก)

In peer-review journals (total impact factor 8.173)

1) Pramoonjago P, Pakdeesuwan K, Siripanyaphinyo U, Chinprasertsuk S, Kinoshita T, **Wanachiwanawin W.** Genotypic, immunophenotypic and clinical features of Thai patients with paroxysmal nocturnal haemoglobinuria. Br J Haematol 1999;105:497-504. (Impact factor 3.195)

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3) Pakdeesuwan K, Muangsup W, Pratya YU, Issaragrisil S, **Wanachiwanawin W.** Clinical paroxysmal nocturnal hemoglobinuria is the result of expansion of glycosyl-phosphatidyl-inositol-anchored protein-deficient clone in the condition of deficient hematopoiesis. Int J Hematol 2001;73:64-70. (Impact factor 1.386)

4) **Wanachiwanawin W**, Siripanyaphinyo U, Piyawattanasakul N, Kinoshita T. A cohort study of the nature of paroxysmal nocturnal hemoglobinuria clones and PIG-A mutations in patients with aplastic anemia. Eur J Haematol 2006:76:502 □ 509. (Impact factor 1.863)

Books or Proceedings

5) **Wanachiwanawin W**, Piyawattanasakul N, Visuthisakchai S, Issaragrisil S. Nature of PNH clones in aplastic anemia. In: Omine M, Kinoshita T (Eds) Paroxysmal nocturnal hemoglobinuria and related disorders: Molecular aspects of pathogenesis. Tokyo: Springer, 2003: 229-33.

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การนำไปใช้ประโยชน์

❑ เชิงสาธารณะ

- โครงการนี้ก่อให้เกิดความร่วมมือทางวิชาการกับนักวิจัยจากมหาวิทยาลัย Osaka ประเทศญี่ปุ่น ทำให้มีโอกาสส่งนักศึกษา PhD, MD และนักวิทยาศาสตร์ไปศึกษาดูงานระหว่างการดำเนินโครงการ และยังเป็นจุดเริ่มต้นของโครงการใหม่ที่กำลังจะเกิดขึ้นกับมหาวิทยาลัย Kyushu ประเทศญี่ปุ่นในโครงการ NRCT-JSPS Asian CORE
- โดยการเผยแพร่องค์ความรู้ที่เกิดขึ้น รวมทั้งการบรรยายในที่ประชุมวิชาการต่างๆ ทำให้โรค PNH เป็นที่รู้จักและสนใจในหมู่แพทย์โดยเฉพาะโลหิตแพทย์เพิ่มขึ้น

❑ เชิงวิชาการ

- การพัฒนาการเรียนการสอน องค์ความรู้ที่เกิดขึ้นใหม่ นี้ใช้ประกอบในการเขียนตำราโลหิตวิทยา สำหรับแพทย์ทั่วไปและโลหิตแพทย์
- สร้างนักวิจัยใหม่ โดยมีนักวิทยาศาสตร์ที่เข้าร่วมโครงการ 2 คน นักศึกษา 2 คน