Peripheral Blood Cells Are Predominantly Chimeric of Affected and Normal Cells in Patients With Paroxysmal Nocturnal Hemoglobinuria: Simultaneous Investigation on Clonality and Expression of Glycophosphatidylinositol-Anchored Proteins

By Haruhiko Ohashi, Tomomitsu Hotta, Atsushi Ichikawa, Tomohiro Kinoshita, Ryo Taguchi, Takeshi Kiguchi, Hiroh Ikezawa, and Hidehiko Saito

To investigate clonal compositions of hematologic cells in paroxysmal nocturnal hemoglobinuria (PNH), we analyzed peripheral blood (PB) cells of 12 female patients with PNH, by clonality analysis using X-chromosome inactivation and assessment of expression of glycophosphatidylinositol-anchored proteins (GPI-APs) by flow cytometry. Southern hybridization showed that granulocytes were monoclonal in three and polyclonal in eight patients, respectively, whereas lymphocytes were polyclonal in all nine patients examined. Expressions of CD16 and CD59 on granulocytes varied greatly in seven patients examined. Clonality analysis of granulocytes by the polymerase chain reaction showed that CD59− and CD59low− cells were monoclonal, whereas CD59+ cells were polyclonal. It was shown that PB cells are predominantly chimeric of clonal (GPI-AP− or GPI-APlow+) and nonclonal (GPI-AP+) cells in PNH, and that degrees of chimerism differ greatly from patient to patient.

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MATERIALS AND METHODS

Patients. Twelve female patients (unique patient number [UPN], 1 to 12) with PNH were studied. All patients were positive for sugar water test and/or acidified serum (Ham) test. Age, duration of disease, presence or absence of precedent aplastic anemia, and PB cell counts at the time of evaluation are summarized in Table 1.
All patients were screened for heterozygosity of three RFLPs for two X chromosome-linked genes: BamH1 RFLP of the hypoxanthine phosphoribosyltransferase (HPRT) gene and those of Bgl I and BstXI of the phosphoglycerate kinase (PGK) gene. Eight of the 12 patients were heterozygous for at least one of these RFLPs. Four patients who were homozygous for all the RFLPs were screened for heterozygosity of polymorphism for VNTR located on the DXS255 locus, for which all proved to be heterozygous.

Cells. Heparinized blood (10 to 20 mL) was obtained from each patient by venipuncture, with informed consent. After sedimentation through Ficoll-Hypaque (Pharmacia, Piscataway, NJ), mononuclear cells (MNCs) were removed from the interface layer. Polymorphonuclear cells (PMNCs) were collected by removal of lysed RBCs after incubation in ammonium chloride buffer. In one patient (UPN 1), PMNCs were also recovered from the cell pellet by agglutination with sheep RBCs (Kyokuto, Osaka, Japan). In some patients, T lymphocytes were separated from MNC fractions by agglutination with sheep RBCs (Kyokuto, Tokyo, Japan). The purity of each cell fraction was affirmed in some samples by morphologic examination of Cytospin preparations. More than 70% of the cells were lymphocytes in MNC fractions, more than 95% were of lymphocytic morphology in T-cell fractions, and more than 95% were granulocytes in PMNC fractions. High-density nuclear cells (MNCs) were removed from the interface layer. Poly

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Duration of disease is the number of years after diagnosis of PNH. Precipitant AA signifies the presence (+) or absence (−) of confirmed history of aplastic anemia before the diagnosis of PNH had been made.

Abbreviations: Hb, hemoglobin; Ret, reticulocyte.

were separated by electrophoresis through 1.0% (for HPRT and DXS255 studies) or 1.5% (for PGK studies) agarose gel (Bio-Rad Laboratories, Richmond, CA) and transferred to nylon membranes (GeneScreen Plus, Biotechnology Systems, Boston, MA). The membranes were hybridized to the probe radiolabeled by the random primer method (Random Primed DNA Labeling Kit; Boehringer Mannheim), washed, and exposed to x-ray films (X-OMAT AR, Eastman-Kodak, Rochester, NY) for 2 to 7 days.

The probe used for PGK studies was 800 bp EcoRI-BamHI fragment containing the 5' region of the PGK gene cloned into pSPT19-1 (provided by Dr J. Singer-Sam, Beckman Institute). For HPRT studies, two probes were used: HPRT-600, 600 bp of Hp II fragment, and HPRT-800, 800 bp of Hp II-Pst I fragment, both of which were subfragments of HPRT 1.7 cloned into PGEM-4 (provided by Dr B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD). For HPRT studies, each membrane was hybridized initially to HPRT-600 probe. When evaluable bands of 18 kb and/or 12 kb did not appear on Hp II treated lanes, it was rehybridized to HPRT-800 probe. M27β probe, used for DXS255 studies, was provided originally from Dr N.J. Fraser (Oxford University, UK). The polymerase chain reaction (PCR) analysis of the PGK gene Clonality analysis by PCR, using BstXI-RFLP of the PGK gene and methylation of two Hp II sites, was performed according to the two-step method established by Gilliland et al.19 with some modification. Briefly, 100 ng of DNA was suspended in 100 µL of buffer and equally divided into two fractions, one cleaved by Hp II and the other left undigested. Each solution was precipitated with ethanol, resuspended in 5 µL of distilled water, and added to 45 µL of buffer (16.6 mmol/L ammonium sulfate, 67 mmol/L Tris-HCl [pH 8.8 at 25°C], 6.7 mmol/L MgCl2, 10 mmol/L 2-mercaptoethanol, 6.7 µmol/L EDTA, 1.5 mmol/L each of 4 deoxyribonucleoside triphosphates, and 170 µg/mL bovine serum albumin) containing the first set of primers (1A and 1B, 1 µmol/L each) and 1 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT), and amplified for 60 cycles (95°C for 1 minute, 55°C for 1 minute, and 74°C for 2 minutes) in a thermal cycler (Perkin Elmer Cetus). One tenth of each reaction solution (5 µL) was added to the second reaction solution containing the second, nested primers (2A and 2B, 1 µmol/L each) and 60 cycles of amplification was performed. After the second reaction, each solution was precipitated with ethanol, resuspended in distilled water, and cleaved by BstXI I, Digested PCR products, one starting from Hp II and the other from untreated DNA for each sample, were separated by electrophoresis through 2% agarose gel and stained with ethidium bromide.

Flow cytometry and cell sorting. Flow cytometric analysis of CD16+ and CD59+ expression on RBCs, granulocytes, and lymphocytes, with and without phosphatidylinositol-specific phospholipase C (PIPLC) treatment, was performed in seven patients and a healthy volunteer with the method described previously.21 Briefly, heparinized blood was centrifuged at 700g for 10 minutes, and RBCs were removed and washed three times with phosphate-buffered saline (PBS). White blood cells (WBCs) were collected from the buffy coat, after removal of contaminating RBCs by ammonium chloride buffer.

Treatment of cells with PIPLC, which cleaves the linkage of diacylglycerol and inositol-phosphate and releases GPI-APs from the membrane, was performed as described.22 RBCs and WBCs were suspended in 0.25 mol/L sucrose containing 10 mmol/L Tris-HCl buffer (pH 7.4) at concentrations of 10^8 and 10^7 cells/mL, respectively. These cell suspensions (1 mL each) were mixed with 1 U (for RBCs) or 2 U (for WBCs) of PIPLC and incubated for 20 minutes at 37°C. After incubation, the suspension was centrifuged at 700g for 10 minutes. Precipitated cells were then washed two times with PBS, and subjected to flow cytometric analysis. PIPLC was purified

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granulocytes, because cells from the normal control show a broad peak for CD59. The possible presence of CD59− and/or CD59low− cells in UPN 3 and 8 cannot readily be denied (Fig 3 and Table 2).

**Clonality in granulocytes with different expression of CD59, as assessed by PCR.** Results of clonality analysis by PCR were assessed using the same principle as those by Southern hybridization analysis (Fig 1). When only one of the two RFLP bands (530 bp and 433 bp) was detected on the *Hpa* II-treated lane, the sample was judged to be monoclonal. When both the bands were seen on the *Hpa* II-treated lane, it was interpreted as polyclonal.

Granulocytes of UPN 3, which proved to be monoclonal by Southern hybridization (Fig 2), consisted of CD59low− and CD59− cells (Fig 4B). Each population was recovered by flow cytometric cell sorting, and was analyzed for clonality by PCR. Both types of granulocytes showed a monoclonal pattern of methylation, whereas T-lymphocytic fraction (CD59+) proved polyclonal (Fig 5A). Granulocytes of UPN 10 showed a polyclonal pattern of methylation by Southern blotting (Fig 2). By PCR, CD59+ granulocytes were polyclonal and CD59− granulocytes were monoclonal (Fig 5B).

**DISCUSSION**

The results of our investigation on PB cells in PNH by Southern hybridization and flow cytometry are summarized as follows: granulocytes were monoclonal in 3 of 11 patients evaluable, and polyclonal in the other 8 patients; granulocytes of 2 of the 3 "monoclonal" patients were lacking in both CD16 and CD59; and those of the third "monoclonal" patient (UPN 3) consisted of CD16+/CD59− cells and CD16−/CD59low− cells. Granulocytes of "polyclonal" patients and lymphocytes of all the nine patients examined were either positive or bimodal (positive/negative) for expression of CD16 and CD59. This is strong circumstantial evidence for postulating that GPI-AP+ and GPI-APlow+ cells are clonal, whereas GPI-AP− cells are nonclonal cells, within each lineage of blood cells.

To verify this hypothesis more directly, we separately recovered granulocytes in different states of CD59 expression (negative, low positive, and positive) from two patients, one with monoclonal and the other with polyclonal granulocytes, by flow cytometric cell sorting. Clonality analysis by PCR of each fraction of cells showed that in the patient with monoclonal granulocytes (UPN 3), both CD59+ and CD59low− cells were monoclonal, and that in the patient with polyclonal granulocytes (UPN 10), CD59+ cells were monoclonal, whereas CD59− cells were polyclonal. This most clearly shows that GPI-AP− cells are clonal cells, and also gives us sufficient reason to consider, together with the results of Southern hybridization and flow cytometry, that GPI-AP− cells are nonclonal. It is also shown that cells expressing fewer GPI-AP molecules, or low positive cells, are clonal cells as well. Because CD59− cells and CD59low− cells of UPN 3 had a common defect in CD16 expression, and also showed the same pattern of X-chromosome inactivation (the allele that bears the *Bst XI* polymorphic site of the PGK gene being inactive), it is likely that the two populations had derived from a common clonal stem cell. Thus,
one may conjecture that the CD24+ granulocytes that showed an unbalanced pattern of X-chromosome inactivation (consistent with monoclonality), reported by Bessler et al.,17 might have been cells with reduced expression of GPI-APs. However, the unbalanced pattern might also have been caused by extreme lyonization in unaffected cells. Although the unequal intensity of the two bands found in the lymphocytic fractions of UNP 8 (Fig 2) or UNP 3 (Fig 5A) and the CD59+ granulocytes of UPN 10 (Fig 5B) might have reflected this phenomenon, the presence of minor clonal populations cannot theoretically be excluded.

Close examination of PB cells of patients with PNH, through simultaneous assessments of clonality and GPI-AP expression, yields insights into the pathophysiology of this disease and that of other hematologic disorders as well. PB cells in PNH are predominantly chimeric of clonal and nonclonal cells. Even in the patients whose granulocytes proved monoclonal and GPI-AP+, GPI-AP+ lymphocytes were detected. The polyclonal results in lymphocytic fractions are in good accordance with the reports on myelodysplastic syndromes (MDS),25,26 and they also serve to guarantee that the monoclonality of granulocytes is indeed an acquired abnormality. However, this does not necessarily imply that the lymphocytic lineage is not affected by PNH clone. The existence of GPI-AP+ lymphocytes in some PNH patients has been shown by several investigators,4,27,28 and in the present study, lymphocytes of one patient (UPN 6) showed clear bimodal expression of CD59 (Table 2). CD59+ lymphocytes of this patient, if separated from CD59+ lymphocytes, may prove to be monoclonal, and it would confirm that PNH defect can arise in the totipotent stem cell level. Our result showing that complete replacement of granulocytes by PNH clone was observed in only three of the patients might be surprising. Moreover, in two patients (UPN 1 and 9), GPI-AP+ cells were found only among RBCs and almost all the granulocytes were GPI-AP+. Because the presence of CD59+ RBCs in PB seems to be the necessary and sufficient condition for complement-mediated hemoly-
The GPI-AP profiles of these two patients are consistent with the diagnosis of PNH. Clonal compositions of PB cells do not appear to differ between the patients with a confirmed history of precedent aplastic anemia (seven patients) and those without it (five patients). From the viewpoint of disease duration, all five patients who were analyzed within 1 year after diagnosis had polyclonal granulocytes, whereas among the other seven patients (2 to 20 years after diagnosis), three patients had monoclonal granulocytes. This seems to reflect the gradual process in replacement of normal cells by PNH clone. However, on the other hand, the fact that two patients with more than 10 years' duration of the disease (UPN 1 and 5) had polyclonal granulocytes suggests that chimerism could be not only a transitional, but also a stable state of hematopoiesis in PNH.

Our study shows that GPI-AP+ cells in patients with PNH are nonclonal cells. Thus, there is reason enough to assume that they are normal, residual cells. In all seven patients examined by flow cytometry, RBCs and granulocytes with normal patterns of GPI-AP expression were apparently reduced in number, a result which could not be readily explained by complement-mediated cell lysis. Therefore, emergence of clonal cells and diminution of normal cells are two independent events that characterize hematopoiesis in PNH. The etiological relation between the two remains to be clarified.

We succeeded in separating clonal cells from heterogeneous, thus polyclonal, granulocytes by flow cytometric cell sorting with anti-CD59 monoclonal antibodies. This observation serves as a reminder that confirmation of polyclonal patterns by RFLP- or VNTR-methylation analysis does not necessarily rule out the existence of minor clonal populations. It also allows one to postulate that comparable chimerism might exist in PB and/or marrow cells in other hematologic disorders that are considered to be clonal.
Occasional reports of polyclonal cases with MDS\textsuperscript{13} or myeloproliferative disorders\textsuperscript{14} might be such examples.

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**REFERENCES**

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