Paroxysmal Nocturnal Hemoglobinuria Clone in Bone Marrow of Patients With Pancytopenia

By Hideki Nakakuma, Shoichi Nagakura, Norihiro Iwamoto, Tatsuya Kawaguchi, Michihiro Hidaka, Kento Horikawa, Tadashi Kagimoto, Tadahiro Shido, and Kiyoshi Takatsuki

The lack of glycosylphosphatidylinositol (GPI)-anchored membrane proteins such as decay-accelerating factor (DAF) and CD59 on blood cells has a diagnostic value in paroxysmal nocturnal hemoglobinuria (PNH). Because PNH often develops in patients with aplastic anemia (AA), we attempted to detect a PNH clone in the bone marrow (BM) of patients with AA and pancytopenia before affected cells were evident in the peripheral blood (PB). We used flow cytometry with monoclonal antibodies against DAF and CD59 for the detection of the clone. Affected cells were observed in the BM of 3 of 7 patients with AA and 1 of 3 patients with pancytopenia of unknown origin, but not in their PB. All 8 patients with apparent PNH had affected cells in their BM and PB. On the basis of the early appearance of the PNH clone in the BM, a prospective 4-month follow-up study of the PB cells was performed. The study showed the release of affected mature cells in granulocytes, then in monocytes, and finally in lymphocytes. Ham's test was positive before affected erythrocytes were detected by flow cytometry. Our findings indicate that detection of the PNH clone in BM could be predictive of the development of PNH in patients with AA and pancytopenia.

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From the Laboratory of Biomembrane Research, The Second Department of Internal Medicine, Kumamoto University School of Medicine, College of Medical Science, Kumamoto University, and National Kumamoto Hospital, Kumamoto, Japan.

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Address reprint requests to Hideki Nakakuma, MD, PhD, The Second Department of Internal Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto 860, Japan.

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weeks after and before the beginning of therapy, respectively. Initial normal erythrocytes and platelets, respectively. 0.3 to 2.0 g/L). Solid and empty arrows indicate transfusions with treatment. As with patient 1, she was similarly diagnosed as having PNH.

**MATERIALS AND METHODS**

Chemicals. Mouse antihuman DAF monoclonal antibody (MoAb; IgG) and its fluorescein isothiocyanate (FITC)-conjugates were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mouse anti-CD2 MoAb (Leu 5b, IgG) and mouse anti-CD19 MoAb (Leu 12, IgG) were obtained from Becton Dickinson Laboratories Inc (Mountain View, CA). Anti-CD13 MoAb (MCS-2, IgG) and phycoerythrin (PE)-conjugated affinity-purified donkey-antimouse IgG were obtained from Nichirei Corporation (Tokyo, Japan) and Jackson Immunoresearch Laboratories Inc (West Grove, PA), respectively. An aliquot of anti-CD59 MoAb was conjugated with FITC. In brief, 10 mg of IgG, was incubated for 6 hours at room temperature in sodium carbonate alkaline buffer (pH 9.5) with 0.9 mg of FITC. The FITC-conjugated IgG (6.4 mg) in phosphate-buffered saline (PBS) was then purified by gel column chromatography. CD2 is a pan-T-cell marker; CD19, a marker of B cells; and CD13, a marker of granulocytes and monocytes.

Preparation of blood cells. After both patients gave their informed consent, heparinized PB cells and BM mononuclear cells were obtained before and after beginning treatment. The same samples were also obtained from healthy adult volunteers and from patients with current PNH, AA, pancytopenia of unknown origin, myelodysplastic syndrome (MDS), and leukemia. An aliquot of whole venous blood was used for the analysis of erythrocytes. Granulocytes were isolated with dextran sulfate and Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Lymphocytes in the PB and mononuclear cells in the BM were isolated by Ficoll-Hypaque centrifugation, as described previously. To improve the purity of immature BM cells, lymphocytes were removed with magnetizable polystyrene beads coated with MoAb to CD2 and CD19 (Dynabeads, Nihon Dynal, Tokyo), as described previously.

Cytfluorometry. Cells were analyzed by flow cytometry, as described previously. In brief, all cells were washed with PBS. Erythrocytes (1 × 10⁸), granulocytes (1 × 10⁹), and BM cells (1 × 10⁹) were incubated on ice with anti-DAF MoAb or anti-CD59 MoAb, and then labeled with FITC-conjugated antimonine IgG. The cells were analyzed with a flow cytometry analyzer (FACScan, Becton Dickinson). Lymphocytes (1 × 10⁹) were incubated with anti-CD2 or anti-CD19 MoAb, labeled with PE-conjugated antimonine IgG, and finally double-labeled with FITC-conjugated MoAb against DAF or CD59. Monocytes in the lymphocyte preparation were labeled similarly to lymphocytes, except that anti-CD13 MoAb was used as the first antibody.

RESULTS

Detection of affected cells with PNH phenotype in BM. Lymphocytes were not detected in the BM preparations by flow cytometry after the lymphocyte exclusion procedure, and virtually all the prepared BM cells were morphologically immature (data not shown). However, the coexistence of native lymphocytes in the BM cells did not exert any practical influence on the cytfluorometric findings. As predicted, all eight patients with PNH had affected cells with the PNH phenotype in their BM (Fig 2C) as well as in the PB (Fig 3C). Subsequently, we also identified affected cells among the mononuclear cells in the BM of four patients who showed pancytopenia, but who had no affected cells detectable in the PB. In two of these four patients, blood cells in the PB were carefully followed by flow cytometry until Ham’s test was positive; however, the other two, who were outpatients, dropped out of the follow-up. Affected cells in their BM were detected 6 to 8 weeks before Ham’s test was positive. That is, patient 1, with AA (Fig 2A), and patient 2, with...
pancytopenia of unknown origin (Fig 2B), showed 9% and 13%, respectively, of BM cells negative for DAF. Because the analysis of BM cells was performed early in the development of PNH, the difference in DAF-negative populations between the patients (Fig 2, A and B) and control (Fig 2D) was, inevitably, small. However, the shape of the curve for the DAF-negative population in the patients was convex, such a curve often indicating PNH cells. Moreover, the population of cells negative for another GPI-anchored protein, CD59, was barely detectable (Fig 2, A and B). The percentages of this population were 2% and 4% in patients 1 and 2, respectively, calculated reproducibly from the numbers of affected cells per 10,000 cells counted. Two weeks after the flow cytometry (Fig 2A), the bone marrow cells in patient 1 were further analyzed by two-color flow cytometry (Fig 3). Affected cells negative for DAF and CD59 were obviously detected despite a low population.

Pathognomonic appearance of affected cells. To confirm the specificity to PNH, we analyzed BM cells obtained from healthy volunteers, from patients with PNH, from those with PNH-related stem cell disorders (AA, MDS, and leukemia, which rarely precede or follow PNH), and from those with pancytopenia of unknown origin. Cells with the PNH phenotype were detected in all eight patients with PNH (Fig 2C), in three of seven patients with AA (Fig 2A), and in one of three patients with pancytopenia of unknown origin (Fig 2B); however, such cells were not detected in the five healthy volunteers (Fig 2D), in the eight patients with leukemia, or in the nine patients with MDS (data not shown). Patients who had affected mononuclear cells in the BM subsequently had affected cells in the PB (Fig 4A and B), and Ham’s test was then positive. To the contrary, those patients who did not have abnormal cells in the BM, even those patients with AA, which is a PNH-predisposing disorder, did not produce PNH cells in the PB. Therefore, the affected cells in the BM were considered to be pathognomonic to the early stage of PNH development.

Advent of affected cells in circulating blood. Steroid therapy for pancytopenia brought about clinical improvement in patient 2, but not in patient 1. However, despite this therapy, LDH activity increased, Ham’s test was then positive in both patients (Fig 1), and affected leukocytes appeared in their PB (Fig 4A and B). The patients were then diagnosed as having PNH. However, we could not detect affected erythrocytes by flow cytometry even 1 month after these hemolytic manifestations. After the first detection of affected cells in the BM of the patients, it was more than 1 month before Ham’s test was positive and more than 2 months before affected circulating erythrocytes were detected. We also noted some cell lineage-dependent time lag in the detection of affected cells, roughly in the order of granulocytes, monocytes, and T lymphocytes (Fig 4, A and B). Eventually, B lymphocytes and erythrocytes were the last to be detected. The analysis of CD59 expression on the PB cells showed similar results to that of DAF, but, as a whole, DAF-deficient cells were detected earlier than CD59-deficient cells in individual cell lineages (data not shown).

DISCUSSION

We first confirmed by flow cytometry the presence of affected cells with the PNH phenotype in the BM of all eight patients already diagnosed as having PNH, and next noted that the affected cells resided latently in the BM of the four patients with pancytopenia (AA and unknown origin) for a considerable time before the conventional hemolysis tests, ie, the sugar-water test and Ham’s test, were positive. Only the patients with affected mononuclear cells in the BM later yielded affected cells in the PB and subsequently had a positive Ham’s test. These patients were, consequently, diagnosed as having PNH. In short, affected stem cell produced affected progenitor cells first in the BM, and affected mature cells were then released into the PB. In general, the typical PNH phenotype is characterized distinctively by the complete deficiency of multiple GPI-anchored membrane proteins on blood cells alone. However, the expression of DAF and CD59 is altered in disorders other than PNH: congenital deficiency of these proteins has been reported, as has decreased expression in both natural killer (NK) and virus-infected cells and maturation-associated changes. To confirm that the cells negative for
DAF and CD59 were associated with PNH, we analyzed the BM cells of patients with PNH, AA as a PNH-predisposing disorder, and other PNH-related stem cell disorders (MDS and leukemia). We confirmed that the affected cells detected in the BM were pathognomonic to PNH and were also predictive of the development of PNH. The detection of affected cells in BM in such a small sample of patients is not surprising because the reported incidence of PNH in PNH-related disorders is moderately high. Thus, the appearance of affected cells in the BM plainly indicates the advantages of BM analysis both for the early diagnosis of PNH and for investigation of the development of PNH in disorders predisposing to this condition.

Regarding the appearance of affected cells in the PB after their appearance in BM, there were some noteworthy findings. (1) The hemolytic manifestations (elevated LDH activity, positive Ham’s test) that indicated the presence of affected erythrocytes in the PB preceded the cytofluorometric detection of these erythrocytes in the PB. The findings suggest that affected erythrocytes appear insidiously in the PB, and that some of these survive complement-mediated hemolysis and accumulate in quantities sufficient to be detected by Ham’s test, but not yet by flow cytometry. Accordingly, the susceptibility of the affected cells to complement possibly defines the preferential detection of the population of affected cells in PB. This idea is supported by a case report showing that, in a PNH patient with coexisting C9 deficiency, hemolytic attacks did not occur, and there was a resultant accumulation of affected cells in the PB. For the early identification of affected cells in PB, other GPI-anchored membrane proteins, such as acetylcholinesterase for erythrocytes, neutrophil-alkaline phosphatase for granulocytes, and CDw52 for lymphocytes, may be better indicators than the complement regulatory membrane proteins DAF and CD59. (2) Affected cells were detected at the early stage among granulocytes, monocytes, and then lymphocytes, but not yet among erythrocytes. After affected granulocytes were detected, but before the detection of affected erythrocytes by flow cytometry, Ham’s test was positive. The preferential detection of affected leukocytes is consistent with previous reports of patients with AA and PNH, although, in those reports, neither affected cells in the BM nor the development-associated appearance of affected cells in the cell lineages were analyzed. Concerning the appearance or detection of affected cells, precise priorities for cell type and GPI-anchored proteins represent an area for further investigation.

PNH is often manifested as a late clonal complication in patients with AA, especially during the clinical remission induced by immunosuppressive therapy with antilymphocyte globulin. Although our patients were not treated with such a strong immunosuppressant, it is likely that the steroid therapy for pancytopenia led to the appearance of PNH by enhancing the proliferation and differentiation of latent PNH stem cell or affected progenitor cells. However, relative to the duration of maturation in normal erythropoiesis, the process from the time of the first detection of affected progenitors in the BM to the diagnosis of PNH took a considerable time, despite the possible stimulation of erythropoiesis by both the therapy and anemia-induced physiologic feedback. The delay in the conventional diagnosis is possibly explained by the intravascular hemolytic exclusion of affected erythrocytes and by the retarded production of affected cells due to the impaired growth and maturation of affected progenitors. This idea is supported by a recent finding that cultured PNH cells showed slower mitogenesis than control cells in vitro.

Based on the concept that the population of affected cells in the PB is regulated by their production in the BM and by their clearance from the PB, principally by complement-mediated cytolysis and natural death, four clinical stages in PNH can be proposed according to the appearance of affected cells: I, affected cells in BM alone; II, affected cells in both BM and PB; III, affected cells in PB alone; IV, affected cells neither in BM nor PB. Thus, we conclude that the analysis of both BM and PB cells not only contributes to the early diagnosis of PNH in PNH-predisposing disorders, but may also serve as a critical probe for the evaluation
of the entire clinical course, including clinical stage, effects of treatment, and prognosis of current PNH.

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