Persistence of Affected T Lymphocytes in Long-Term Clinical Remission in Paroxysmal Nocturnal Hemoglobinuria

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Long-term clinical remission of more than 10 years is rarely seen in paroxysmal nocturnal hemoglobinuria (PNH). Affected blood cells in PNH lack glycosylphosphatidylinositol (GPI)-anchored membrane proteins such as decay-accelerating factor (DAF) and CD59. We performed a flow cytometric analysis of circulating blood cells obtained from two patients with PNH who had been in clinical remission for more than 10 and 25 years, respectively. Affected cells with the PNH phenotype were demonstrated only among T-lymphocytes. Persistent affected T cells were negative for the CD52 protein only, this protein being a GPI-anchored lymphocyte marker without complement regulatory activity. The persistence of the affected T cells may be explained either by an inherently long life span after the disappearance of the PNH stem cell or by insidious production at a subclinical level by affected stem cell. In either event, detection of affected T cells, especially CD52-negative T cells, may be useful for the evaluation of long-term clinical remission in PNH.

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PATIENTS with paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic disease arising from a stem-cell disorder, often exhibit various complications, such as venous thrombosis and bone marrow dysplasia.1,2 The hemolytic features are due to the abnormal susceptibility of affected cells to autologous complement.3 This increased sensitivity has been attributed to a deficiency of complement regulatory membrane proteins, such as decay-accelerating factor (DAF; CD55) and CD59, which are localized on the cell membrane via a glycosylphosphatidylinositol (GPI) anchor.4 Recent studies have elucidated the molecular mechanism responsible for the abnormal hemolysis by showing a synthesis defect in the carbohydrate moiety of the anchor,5 by determining the interruption site in anchor synthesis,6 and by identifying the gene responsible for the impaired synthesis.7 Thus, the lack of GPI-anchored membrane proteins is of diagnostic value in PNH. Long-term clinical remission is rarely noted in PNH, as it is in other acquired clonal disorders.8 Although remission is not necessary for survival in PNH patients, it is generally considered that the symptoms and clinical manifestations characteristic of PNH disappear in clinical remission; however, the laboratory findings in PNH remission have not yet been well defined. In the present study, using flow cytometry, we showed the persistence of affected T cells with the PNH phenotype in two patients with PNH in clinical remission for more than 10 years.

PATIENT PROFILES

During the last 30 years, we have treated 38 patients with PNH in our hospital. Three of these patients have been in long-term clinical remission (more than 10 years). In two of these patients we were able to analyze the expression of GPI-anchored proteins on peripheral blood cells. Table 1 shows the relevant laboratory data of the two patients. PNH was diagnosed on the basis of the history, clinical findings, Coombs’ test—negative intravascular hemolysis, and acidified serum test (Ham’s test).

Patient 1. In 1982, a 45-year-old woman presented with progressive anemia. Her hemogram showed pancytopenia. However, clinical and laboratory findings did not satisfy the criteria for the diagnosis of aplastic anemia (AA), PNH, or other disorders in which pancytopenia is often manifested. Although the diagnosis was not determined, she was administered mepitiostane and then prednisolone to treat the progressive anemia. Five months later, the patient felt better and the hemogram showed an improvement. However, serum lactate dehydrogenase (LDH) activity was elevated. Later, results of both the sugar-water test and Ham’s test were positive. She was then diagnosed with PNH. Because she showed a good response to the treatment and was free of serious complications, the steroids were gradually tapered off, eventually being discontinued 11 months after the beginning of therapy. Even after the steroids were discontinued, the patient’s hemogram slowly but steadily improved. Her serum LDH titer also returned to the normal range. She has since had none of the symptoms often seen in PNH, and she has been in remission for more than 10 years. During remission, results of the Ham’s test were confirmed to be negative.

Patient 2. In 1965, a 24-year-old woman in our institution was diagnosed with AA, based on the presence of pancytopenia and hypoplastic bone marrow without atypical cells. She needed frequent blood transfusions until steroid treatment (oxymetholone and betamethasone) induced clinical improvement. The steroids were then tapered off, eventually being discontinued in May 1967. Although her condition had improved, results of the Ham’s test and sugar-water test were positive in October 1967, and neutrophil alkaline phosphatase (NAP) gradually decreased. She was then diagnosed with PNH, but was free of severe complications. She later exhibited spontaneous clinical remission, which has now continued for more than 25 years. In remission, results of hemolysis tests have been negative. Of note, mild intravascular hemolysis was suggested by hemosiderinuria and an increased serum LDH titer.

MATERIALS AND METHODS

Chemicals. Mouse antihuman DAF monoclonal antibody (MoAb, IgG1) and its fluorescein isothiocyanate (FITC) conjugates

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Table 1. Relevant Laboratory Data in Patients With PNH in Long-Term Clinical Remission

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (10^9/L)</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Erythrocytes (10^12/L)</td>
<td>2.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Platelets (10^12/L)</td>
<td>47</td>
<td>64</td>
</tr>
<tr>
<td>LDH*</td>
<td>373</td>
<td>611</td>
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<tr>
<td>Haptoglobin (g/L)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Hemosiderinuria</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ham's test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sugar-water test</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

* Lactate dehydrogenase activity in serum (normal range, 236–427 W).
† Serum haptoglobin level (normal range, 0.3–2.0 g/L).

were purchased from Wako Pure Chemical Industries (Osaka, Japan); mouse antihuman CD59 MoAb (IgG3) was a gift from Dr Motowo Tomita of Showa University, Tokyo. FITC-conjugated anti-CD5 MoAb (CAMPATH-1H) was donated by Nippon-Welldone (Osaka, Japan). FITC-conjugated goat antimouse IgG was obtained from Zymed Laboratories Inc (San Francisco, CA). Mouse anti-CD2 MoAb (Leu 5b, IgG2a), anti-CD3 MoAb (Leu 4, IgG3), and anti-CD19 MoAb (Leu 12, IgG1) were obtained from Becton Dickinson Laboratories Inc (Mountain View, CA). Anti-CD13 MoAb (MCS-2, IgG1) and phycoerythrin (PE)-conjugated affinity-purified donkey antimouse IgG were obtained from Nichirei Corporation (Tokyo) and Jackson Immunoresearch Laboratories Inc (West Grove, PA), respectively. FITC-conjugated anti-CD59 MoAb was prepared as described previously. In brief, 10 mg of IgG, was incubated for 6 hours in sodium carbonate alkaline buffer (pH 9.5) with 0.9 mg of FITC, and the FITC-conjugated IgG (6.4 mg) was then purified in phosphate-buffered saline (PBS) by gel column chromatography. The cell-surface markers used were as follows: CD2 and CD3, T-cell markers; CD19, a B-cell marker; CD13, a granulocyte and monocyte marker; and CD52, a GPI-anchored lymphocyte marker that is not a complement regulatory protein.

Preparation of blood cells. In 1993, we obtained heparinized venous blood samples from five healthy volunteers, the two patients in long-term remission of PNH, and seven patients with current PNH. All subjects gave their consent to the procedure. An aliquot of whole venous blood was used for the analysis of erythrocytes. Granulocytes were isolated with dextran sulfate and Ficoll-Conray (Pharmacia, Uppsala, Sweden). Circulating lymphocytes and monocytes were obtained by Ficoll-Conray centrifugation, as described previously.

Cytocentrifugation. Cells were analyzed by flow cytometry, as described previously. In brief, the cells were washed with PBS. Erythrocytes (1 × 10^8) and granulocytes (1 × 10^8) were incubated on ice with anti-DAF MoAb or anti-CD59 MoAb, and then labeled with FITC-conjugated antihuman IgG. Lymphocytes (1 × 10^7) were incubated with anti-CD2, anti-CD3, or anti-CD19 MoAb, then labeled with PE-conjugated antimouse IgG and further labeled with FITC-conjugated MoAb against DAF, CD59, and CD52. Monocytes in the lymphocyte fraction were labeled with anti-CD13 MoAb, then with PE-conjugated second antibody, and finally with FITC-conjugated MoAb against DAF or CD59. Labeled cells were analyzed with a flow cytometry analyzer (FACScan, Becton Dickinson).

RESULTS AND DISCUSSION

Figure 1 shows the expression of DAF on circulating blood cells obtained from the two patients with PNH in long-term clinical remission (Fig 1A, patient 1; Fig 1B, patient 2), from representative samples of the seven patients with PNH (Fig 1C), and from the five healthy volunteers (Fig 1D). Because results for granulocytes and monocytes were similar, we regarded monocytes as being representative of both cell types. Control cells obtained from the healthy volunteers were clearly positive for DAF, except for T-lymphocytes, which showed a wide range of DAF expression from
weakly positive to clearly positive, as described previously (Fig 1D)\(^ {10,11}\). On the other hand, all patients with current PNH showed various populations of affected cells negative for DAF in every cell lineage (Fig 1C), whereas the two patients in clinical remission had affected cells only among the T-lymphocytes (Fig 1A and B). The expression of CD59 on blood cells was similar to that of DAF (data not shown).

To confirm the presence of affected T cells with the PNH phenotype, we subsequently demonstrated affected T cells negative for two other GPI-anchored proteins, CD59 and CD52; such affected cells were clearly detected in patient 1 (Fig 2A) and were detected, but to a lesser extent, in patient 2 (Fig 2B). Two-color analysis showed the presence of affected cells negative for at least two of the three GPI-anchored proteins, that is DAF, CD59, and CD52, a pattern suggestive of typical PNH lymphocytes (Fig 3). These results suggest that PNH T cells had persisted during the long-term clinical remission of more than 10 and more than 25 years. In March 1994, we performed a second analysis of blood cells in patient 1. Flow cytometry showed the distinct persistence of affected T cells negative for CD52 alone (Fig 4).
These cells were positive for CD2, CD3, DAF, and CD59, suggesting that they had the normal resistance to complement. Throughout the study, no B cells with PNH phenotype were detected, and other GPI-anchored proteins, ie, CD14 on monocytes and NAP on granulocytes, were expressed normally in the two patients in clinical remission (data not shown).

Accordingly, we noted the distinct persistence of affected T-lymphocytes in long-term clinical remission in PNH; one possible explanation for this persistence is that these T-lymphocytes have an inherently long life span after the PNH stem cell has disappeared. Indeed, we found that the population of affected T cells was lower in the patient who had been in remission for more than 25 years than in the patient whose remission had lasted for more than 10 years. Another explanation is that the PNH stem cell lies latent in the bone marrow and insidiously (subclinically) yields affected cells during remission. This possibility is supported by the findings of intravascular hemolysis. That is, urine hemosiderin and moderately high serum LDH titers were confirmed in that work. Our findings suggest that CD52-negative T lymphocytes could serve as a distinct marker for the follow-up of clinical remission in PNH. Because the clinical and laboratory signs of PNH disappear in complete remission, more intensive investigation by flow cytometry and by cytogenetic analysis of the PIG-A gene should provide a better understanding of the nature of long-term clinical remission in this disease.

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