Genetic Defects Underlying Paroxysmal Nocturnal Hemoglobinuria That Arises Out of Aplastic Anemia

By Shanmugam Nagarajan, Robert A. Brodsky, Neal S. Young, and M. Edward Medof

Treatment of severe aplastic anemia with antithymocyte globulin (ATG) and cyclosporin leads to clinical remission in a large proportion of patients. As many as 10% to 57% of these patients, however, develop paroxysmal nocturnal hemoglobinuria (PNH). We and others have observed that this secondary PNH appears to be more indolent than classical PNH, which results from an acquired mutation in the PIG-A gene. In the present study, we compared PIG-A mRNA transcripts in affected cells from patients with secondary PNH and patients with classical PNH. All four of our aplastic patients who developed PNH had a negative Ham test at diagnosis. Two of the four showed a positive Ham test within 3 months after ATG/cyclosporin administration, one developed a positive test at 6 months, and another at 18 months after immunosuppressive therapy. All four patients remain transfusion-independent with no thrombotic episodes after a mean follow-up of 30 months (range, 6 to 63 months). Reverse transcription-polymerase chain reaction (RT-PCR) of PIG-A transcripts in DAF/CD59- neutrophils or lymphocyte lines of the four patients showed PIG-A abnormalities in all cases. Transition of C<sup>465</sup> to T was found in one, a 14-bp deletion (positions 1141 to 1154) was found in the second, deletion of C<sup>49</sup> was found in the third, and two mutations, transition of C<sup>469</sup> to T and transversion of T<sup>470</sup> to A, were found in the fourth. These abnormalities compared with findings of abnormal RNA splicing causing a 133-bp deletion, a 4-bp insertion (between positions 578 and 579), loss of A<sup>575</sup>, and loss of C<sup>579</sup> in four patients with primary PNH. We conclude that secondary PNH that evolves out of aplastic anemia, like classical PNH, is associated with mutations in the PIG-A gene. The apparent indolent nature of this disease probably reflects early detection.

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MATERIALS AND METHODS

Immunoreagents. Murine anti-decay accelerating factor (DAF) monoclonal antibody (MoAb) IA10 was prepared as described. Murine anti-CD59 MoAb IF5 and anti-CD48 MoAb were provided by Dr H. Okada (Nagoya City University, Nagoya City, Japan) and Dr J. Pesando (Biomembrane Institute, Seattle, WA). Murine anti-CD20 and anti-CD21 were purchased from Cappel (West Chester, PA). Murine anti-decay accelerating factor (DAF) MoAb were purchased from The Binding Site (San Diego, CA). Murine RCP5 nonrelevant IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were purchased from Cappel (West Chester, PA).

Patients. The clinical features of the aplastic anemia patients who developed secondary PNH are summarized in Table 1. All four patients met the criteria for severe aplastic anemia. Each patient was treated with ATG (40 mg/kg/d) for 4 consecutive days and CsA starting at 12 mg/kg/d. The CsA dose was adjusted for nephrotoxicity and blood levels every 2 weeks for a total of 6 months. Each patient showed a negative Ham test at the time of aplastic anemia diagnosis but developed a positive Ham test 3 to 18 months after completion of treatment. All patients were asymptomatic at the time of their PNH diagnosis. Only one patient, B.E., required a blood transfusion but, since that time, has been transfusion-independent. One patient (B.H.) developed symptoms (symptomatic anemia) after diagnosis. The remaining patients remain asymptomatic with mild peripheral pancytopenia.

The four control patients with classical PNH were diagnosed by Ham test, anti-DAF/CD59 staining of blood elements, and bone marrow examination. Each showed a large proportion of PNH III erythrocytes (as assessed by cobra venom factor bystander lysis).
and greater than 95% DAF/CD59- polymorphonuclear leukocytes (PMN).

Preparation of (GPI-deficient) affected cell populations. Affected PMN (S.T., A.P., and S.S.), Epstein-Barr virus (EBV)-transformed affected B cell lines (J.V.-, L.D.-, and B.H.-), or affected T cell lines (M.W.-) were used as a source of RNA.

For the preparation of affected PMN, cells were isolated by Ficoll-Hypaque centrifugation (Pharmacia, Piscataway, NJ) followed by dextran sedimentation. After removal of contaminating erythrocytes by hypotonic lysis, cells were depleted of unaffected PMN using dextran sedimentation. After removal of contaminating erythrocytes (PMN).

Coll-Hypaque-separated mononuclear cells were incubated for 3 hours at 37°C with EBV supernatant. After 24 hours of further incubation, the infected cells were enriched for affected cells by absorption with anti-CD48 MoAb and goat anti-murine IgG immunoglobulins (Dy-nal Inc, Lake Success, NY). Greater than 90% homogeneity of affected cells was verified by flow cytometric analysis of the adsorbed cells after staining with 1A10 anti-DAF or IF5 anti-CD59 MoAb and FITC-labeled anti-murine Ig.

For the preparation of EBV-transformed B lymphocyte lines, Ficoll-Hypaque-separated mononuclear cells were incubated for 3 hours at 37°C with EBV supernatant. After 24 hours of further incubation, the infected cells were enriched for affected cells by absorption with anti-CD48 MoAb and goat anti-murine IgG immunoglobulins (Collaborative Research Inc, Bedford, MA). The enriched cells were cultured at 10^4 cells per well in 96-well plates. After expansion, individual clones were analyzed by flow cytometry, and CD48^- (and CD48+) lines were purified to homogeneity by sorting. Transformed B cell lines were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 medium (GIBCO-BRL, Grand Island, NY) supplemented with 20% heat inactivated fetal bovine serum, 5 µg/mL insulin, and 5 µg/mL transferrin.

In the case of T cell lines, CD48^- and CD48+ enriched lymphocytes were grown on irradiated L929 feeder layers in the presence of lymphocyte-conditioned medium as described. After expansion, CD48^- and CD48+ lymphocytes were selected with anti-CD48 MoAb and goat anti-murine IgG immunoglobulins, and the two subsets were reexpansioned homogenous populations of CD48^- and CD48+ lymphocytes were obtained.

Control cell lines, eg, Hela and unaffected CD48^- EBV-transformed B cells from L.D. and an additional patient (D.O.) with primary PNH, were maintained in RPMI/10% heat inactivated newborn calf serum containing 2 mmol/L glutamine.

Flow cytometry. Cells (10^6) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% NaN₃ were incubated with 5 µg/mL specific MoAb or with nonrelevant isotype control followed by FITC-labeled sheep anti-Ig as described. The stained cells were analyzed in a FacSTARPLUS flow cytometer (Becton Dickinson, San Diego, CA).

Reverse transcription (RT)-polymerase chain reaction (PCR) and sequencing. Total cellular RNA was isolated by the guanidium thiocyanate method. The RNA was reverse-transcribed with RNAse H-free murine Moloney leukemia virus reverse transcriptase (Gibco-BRL) in a 20-µL volume containing 200 ng of primer 3′ (5′-AATGATATAAGGTAGGCTAC-3′) and 5′ (5′-CCCCAAAAGCAGTTTATT-3′), 200 µmol/L deoxyribonucleotide triphosphates (dNTPs), and 1 µL Taq Polymerase (Promega Biotec, Madison, WI) then were added to 2 µL of the reverse-transcribed product in 2 mmol/L MgCl₂. After incubation for 5 minutes at 95°C, the mixture was amplified by 35 cycles of 1 minute at 93°C, 1.5 minutes at 55°C, and 1 minute at 72°C, followed by a final cycle of 7 minutes at 72°C.

PCR-amplified products were subcloned into pCRITA vector (Invitrogen Inc, San Diego, CA). Inserts were sequenced by the dideoxy DNA method using vector- and PIG-A-specific primers.

RESULTS

Analyses of PIG-A mRNA in affected cells. The products recovered from RT-PCR amplifications of the PIG-A coding region in control and patient cells are shown in Fig 1. Figure 1A shows a diagrammatic representation of the products that are predicted in unaffected cells. Figure 1B shows the products generated from the four aplastic anemia patients with secondary PNH in relation to those generated from normal cells and from the four patients with primary PNH.

As described elsewhere, RT-PCR amplification of the PIG-A genome in normal (PIG-A-sufficient) cells yields three cDNA products of 1,600, 1,200, and 950 bp, as seen for Hela cells (Fig 1B, lane 1) and unaffected B cells derived from patients D.O. (lane 2) and L.D. (lane 3). The larger 1,600-bp product corresponds to transcripts encoding the full-length coding region (1,452 bp). The smaller-sized 1,200-bp and 950-bp products correspond to nonfunctional, alternatively spliced transcripts that lack 374-bp (from position 342 to 715 bp) and 658-bp (from position 58 to 715 bp) internal segments of the coding region that derive from downstream portions of exon 2 of the PIG-A gene.

In the case of RT-PCR products generated from affected cells of the patients with secondary PNH (Fig 1B, right panel), all four patients showed all three PIG-A products (1,600, 1,200, and 950 bp). Although the PCR amplifications were not normalized, in one patient (B.E.), the proportion of 1,600-bp product relative to the 1,200-bp and 950-bp products was decreased. In two of the other patients (B.H. and S.S.), the proportion of the 1,200-bp or 950-bp products was selectively increased, and additional small products were seen.

With regard to the RT-PCR products generated from affected cells of the patients with primary PNH (Fig 1B, middle panel), two patients, J.V. and M.W., showed the three normal-sized PCR-amplified PIG-A products, and two patients showed abnormal-sized products. In one of the two patients with normal-sized products (J.V.), the proportion of the 1,600-bp product was reduced. In one of the two patients with abnormal products (J.V.), products with slightly faster...
mobility were seen, whereas in the other (L.D.), two reduced-sized products of approximately 1,100 and 800 bp were seen.

**PIG-A mutations in secondary PNH are heterogeneous and resemble those in primary PNH.** In each of the above patients, the full-length (1,600 bp) PIG-A product was recovered and subcloned into pCRIIIA vector, and its nucleotide sequence was analyzed. The results of these analyses are summarized in Table 2.

In the case of the patients with aplastic anemia and secondary PNH, one patient (A.P.) showed a deletion of C. This deletion resulted in a frameshift that generated a premature stop codon at 179. The second patient, B.H., showed a transition of C to T. This transition introduced an in-frame stop codon in place of Q. The third patient, S.S., showed a 14-bp deletion extending from position 1141 to 1154 bp. This alteration generated a stop codon 28 bp downstream from the deletion. The fourth patient, B.E., showed two mutations: the first, a transition of C to T, resulting in the replacement of R with a W, and the second, a transversion of T to A, introducing an in-frame stop codon (TAA) in place of Y.

With respect to the patients with primary PNH, two patients, M.W. and S.T., showed single nucleotide deletions of A and C, respectively. These mutations resulted in frameshifts that generated premature stop codons (TAA in each case) at positions 770 and 582, respectively. In patient J.V., a 4-bp (ATGT) insertion was found between positions 78 and 79. A frameshift caused by this insertion introduced a premature stop codon (TGA) at position 604. PCR amplification of genomic DNA using primers corresponding to the 3' end of intron I and the 5' end of intron 2 showed this alteration in three independent clones. In patient L.D., sequence analysis of the largest (1,100 bp) PIG-A PCR product revealed the absence of 507 bp of sequence extending

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**Table 2. PIG-A Mutations in Idiopathic PNH and PNH Evolving From Aplastic Anemia**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Affected Neutrophils (%)</th>
<th>Mutation</th>
<th>Type</th>
<th>Position</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/PNH</td>
<td></td>
<td>Deletion</td>
<td>C</td>
<td>Frame shift TGA&lt;sup&gt;178&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>A.P.</td>
<td>&gt;95</td>
<td>Exchange</td>
<td>C&lt;sup&gt;62&lt;/sup&gt;, T&lt;sup&gt;756&lt;/sup&gt;, A</td>
<td>R&lt;sup&gt;109&lt;/sup&gt; to W, Y&lt;sup&gt;724&lt;/sup&gt; to TAA</td>
<td></td>
</tr>
<tr>
<td>B.E.</td>
<td>68</td>
<td>Exchange</td>
<td>C&lt;sup&gt;65&lt;/sup&gt;, T&lt;sup&gt;756&lt;/sup&gt;, A</td>
<td>T&lt;sup&gt;109&lt;/sup&gt; to TGA</td>
<td></td>
</tr>
<tr>
<td>B.H.</td>
<td>14</td>
<td>Deletion</td>
<td>14 bp&lt;sup&gt;1441-1554&lt;/sup&gt;</td>
<td>TGA&lt;sup&gt;782&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>S.S.</td>
<td>&gt;95</td>
<td>Insertion</td>
<td>ATGT&lt;sup&gt;926-928&lt;/sup&gt;</td>
<td>Frame shift TGA&lt;sup&gt;604&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PNH</td>
<td></td>
<td>Splice defect</td>
<td>GT-AT&lt;sup&gt;926-928&lt;/sup&gt;</td>
<td>Truncated protein (292 aa)</td>
<td></td>
</tr>
<tr>
<td>J.V.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>A&lt;sup&gt;927&lt;/sup&gt;</td>
<td>Frame shift TAA&lt;sup&gt;728&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>I.D.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>C&lt;sup&gt;65&lt;/sup&gt;</td>
<td>Frame shift TAA&lt;sup&gt;682&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>M.W.</td>
<td>40</td>
<td>Deletion</td>
<td>A&lt;sup&gt;927&lt;/sup&gt;</td>
<td>Frame shift TAA&lt;sup&gt;728&lt;/sup&gt;</td>
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</tr>
<tr>
<td>S.T.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>C&lt;sup&gt;65&lt;/sup&gt;</td>
<td>Frame shift TAA&lt;sup&gt;682&lt;/sup&gt;</td>
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PNH GENETIC DEFECTS IN APLASTIC ANEMIA

THIS SERIES

OTHER INVESTIGATIONS

Fig 2. Spectrum of PIG-A mutations in different patients with PNH. The mutations in the patients with secondary and primary PNH analyzed in this series are shown in relation to those found in other investigations. The mutations in the patients with secondary PNH are highlighted in bold. D, deletion; S, base pair exchange (missense or nonsense); I, insertion; L, splicing defect.

from position 342 to 848 in the normal 1,600-bp PIG-A sequence. Comparison of this sequence with the normal 1,200-bp PIG-A splice product (see Fig 1A) showed that the missing sequence encompassed the 374-bp long alternatively spliced (exon 2) segment normally absent in the 1,200-bp product, but included an additional 133 bp of downstream sequence corresponding to exon 3 of the PIG-A gene. Analyses of genomic DNA from patient L.D. showed that the G in the GT donor signal immediately downstream of exon 3 was mutated to an A.

The types and positions of mutations in the patients with secondary PNH arising out of aplastic anemia are shown diagrammatically in relation to those in the patients with primary PNH and those reported in other investigations in Fig 2.

DISCUSSION

In this study, we analyzed the molecular defects in affected cells of four patients with primary PNH and four patients with secondary PNH presenting in the course of treatment and recovery from aplastic anemia. We found that, like patients with primary PNH investigated in this study and elsewhere (see below), patients with secondary PNH uniformly exhibit mutations in the PIG-A gene.

Two of the four patients examined with secondary PNH showed deletions, and the other two showed transitions/transversions. Of the two with deletions, one involved a single nucleotide (C39), and the other encompassed 14 nucleotides (T114 to T115). Of the two transitions/transversions, one consisted of a single C163 to T change, while the other involved two widely spaced changes of C35 to T and T762 to A. In all cases, the alterations resulted in premature stop codons. No consistent pattern of differences were apparent when these mutations were compared with those found in four patients with primary PNH that were studied in parallel. Among these latter patients, two showed single nucleotide deletions (A787 and C795), one a four-nucleotide insertion (ATGT), and one a defect in splicing. In three of these individuals, as in the patients with secondary PNH, the mutations resulted in frameshifts that caused premature stop codons, while in the fourth, the mutation gave rise to a truncated protein.

PIG-A defects have been characterized in a total of 23 other patients with primary PNH to date.5,13-18 As shown in Fig 2, the PIG-A defects in the patients analyzed in this study resemble those found in other patients that have been examined in that they involve mostly small mutations that are randomly situated throughout the PIG-A genome. Among these other patients, six showed single- or two-nucleotide deletions, seven showed transitions/transversions, three showed two-nucleotide to one-nucleotide exchanges, three showed one- to five-nucleotide insertions, and the remainder (four) showed splicing defects. Included among these other analyzed cases are fifteen Japanese, four English, and four American patients. The patients analyzed in this series add an additional seven Americans and one Dominican patient. Collectively, the data argue that there are no hot spots in the PIG-A genome and that the mutations that underlie both secondary and primary PNH arise randomly.

We and others have observed that the clinical courses of patients with secondary PNH appear to be more indolent than those of patients with de novo PNH. All four of the secondary PNH cases investigated in this series were diagnosed exclusively by laboratory tests while they were free
of clinical manifestations (Table 1). One patient developed fatigue and exertional dyspnea after diagnosis, while the others had no clinical abnormalities and remained asymptomatic. Tichelli et al6 reported that 4 of their 13 patients with aplastic anemia who developed PNH after immunosuppressive therapy similarly were diagnosed while they were free of clinical manifestations. Furthermore, a French group2 reported that 6 of 12 patients with aplastic anemia who developed secondary PNH had no clinical symptoms; the remaining six had symptoms relating only to hemolysis. Our finding that affected cells from patients with secondary PNH harbor comparable genetic lesions to those in affected cells from patients with primary PNH argues that any clinical differences between the groups may relate to earlier diagnoses in the patients with secondary PNH.

Aplastic anemia usually arises idiomatically but sometimes develops in association with drug toxicity, viral infection, pregnancy, or other clinical events. The cause of the persistent marrow failure that characterizes the disorder is unknown. A large body of data indicates that the growth of hematopoietic progenitor cells is suppressed by immune mechanisms. In support of this proposal, a high proportion of patients, like those investigated in this study, show dramatic responses to immunosuppressive therapy. The mechanism leading to the appearance of PNH spontaneously in aplastic anemia during the recovery phase from treated aplastic anemia is unknown. One possibility is that the frequency of mutations that disrupt GPI anchor assembly is somehow increased. The fact that the X-linked PIG-A gene is most vulnerable among GPI anchor pathway genes, as documented in this study, is expected, as it is the only single copy gene. An alternate possibility is that the mutation frequency of PIG-A is not increased in this setting but rather expansion of affected PIG-A clones is favored due to diminished competition from normal (unaffected) hematopoietic progenitors, or due to intrinsically better growth in the abnormal environment of aplastic anemia. The finding in the present study that the PIG-A mutations that give rise to PNH in aplastic anemia are indistinguishable from those that underlie primary PNH is consistent with this latter formulation, although it doesn’ t exclude the former possibility of increased mutation frequency. Quantitations of the frequencies of PIG-A mutations in hematopoietic stem cells in normal and aplastic marrows could help to distinguish between these possibilities.

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