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 HEMOLGBINURIA (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 C430 TO T WAS FOUND IN ONE, A 14-BP DELETION (POSITIONS 1141 TO 1154) WAS FOUND IN THE SECOND, DELETION OF C59 WAS FOUND IN THE THIRD, AND TWO MUTATIONS, TRANSITION OF C298 TO T AND TRANSVERSION OF T299 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 A322, AND LOSS OF C326 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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ACQUIRED APLASTIC ANEMIA IS A RARE HEMATOPOIETIC DISORDER CHARACTERIZED BY HYPOCURRENCE OF MARROW IN COMBINATION WITH PERIPHERAL BLOOD PANCYTOPE尼亚. IMMUNOSUPPRESSIVE TREATMENT WITH CYCLOSPORIN A (CSA) AND HORSE ANTITHYMOCYTE GLOBULIN (ATG) OR WITH ANTI-THORACIC DUCT LYPHOCYTE GLOBULIN LEADS TO A CLINICAL REMISSION (ABSOlUTE NEUTROPHIL COUNT [ANC] TO >500/µL) IN 50% TO 70% OF PATIENTS. 2,3 IN CASES WITHOUT PRIOR TREATMENT OR AFTER RECOVERY FROM TREATMENT, HOWEVER, A PROPORTION OF SUCCESSFULLY TREATED PATIENTS DEVELOP CLONAL DISORDERS INCLUDING PAROXYSMAL NOCTURNAL HEMOLGBINURIA (PNH), MYELODYSPLASTIC SYNDROME (MDS), OR ACUTE MYELOGENIC LEUKEMIA (AML). 4 ON EXTENDED OBSERVATION, AS MANY AS 10% TO 57% OF TREATED INDIVIDUALS DEVELOP THE FIRST COMPICATION OF PNH. 5,6 IN A RECENT SURVEY OF 52 PATIENTS' BLOOD CELLS BY FLOW CYTOMETRY, 27 (52%) SHOWED EVIDENCE OF PNH CELLS. 7 IDIOPATHIC OR PRIMARY PNH IS CAUSED BY SOMATIC MUTATION OF AN X-LINKED GENE TERMED PIG-A. 8 THE PRODUCT OF THIS GENE IS NEEDED FOR THE FIRST STEP OF GLYCOSYL-INOSITOL PHOSPHO-LIPID (GPI) ANCHOR BIOSYNTHESIS. 8,10 PIG-A DEFICIENCY RESULTS IN THE FAILED EXPRESSION OF ALL GPI-ANCHORED PROTEINS ON AFFECTED CELL SURFACES. 11 ALL PRIMARY PNH PATIENTS THAT HAVE BEEN EXAMINED TO DATE HAVE SHOWN MUTATIONS OF THE PIG-A GENE, ALTHOUGH THE SITE OF THE DEFECT HAS BEEN DIFFERENT IN EACH CASE. 8,12,13

BASED ON THE SOMETIMES SMALL NUMBER OF AFFECTED CELLS AT DIAGNOSIS, APPARENTLY SLOW PROGRESSION OF THE DISEASE, AND LOW FREQUENCY OF CLINICAL COMPLICATIONS, IT HAS BEEN SUGGESTED THAT SECONDARY PNH THAT EVOLVES OUT OF APLASTIC ANEMIA MAY BE A MORE BENIGN OR INDOLENT DISORDER THAN CLASSICAL PNH. IN THIS STUDY, WE EXAMINED PIG-A mRNA TRANSCRIPTS IN AFFECTED CELS FROM PATIENTS WITH SECONDARY PNH PRESENTING DURING RECOVERY FROM APLASTIC ANEMIA TO DETERMINE IF PIG-A MUTATIONS UNDERLIE THIS COMPLICATION AND IF SO, WHETHER THE GENETIC ALTERATIONS DIFFER FROM THOSE IN CLASSICAL PNH.

MATERIALS AND METHODS

Immunoreagents. Murine anti-decay accelerating factor (DAF) monoclonal antibody (MoAb) 1A10 was prepared as described. 11 Murine anti-CD59 MoAb IF3 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 The Binding Site (San Diego, CA). Murine RPC5 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., B.E., 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, 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 immunobeads (Dynal 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.

In the case of T cell lines, CD48– and CD48+ enriched lymphocytes were obtained.

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Transformed B cell lines were maintained in RPMI/10% heat-inactivated newborn calf serum, 2 mM/L glutamine, 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 immunobeads, and the two subsets were reexpanded until homogenous populations of CD48– and CD48+ lymphocytes were obtained.

Control cell lines, eg, Hela and uninfected 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 mM/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 using the guanidinium 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'-AATGATATAGGGTGAGCAATC-3'), 50 picomoles of amplification primers 1 (5'-CCGTTAATTAGGAGACACATC-3') and 2 (5'-CCCCAAAAGCAAGGTTATT-3'), 200 μM/L dideoxynucleotide triphosphates (dNTPs), and 1 μL Taq Polymerase (Promega Biotec, Madison, WI) then were added to 2 μL of the reverse-transcribed product in 2 mM/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 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 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 (S.T.), products with slightly faster mobility were also observed.

Table 1. Clinical Features of Patients With Aplastic Anemia Who Converted to PNH After Immunosuppressive Therapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ham Test at Diagnosis</th>
<th>Treatment</th>
<th>Interval to Positive Ham Test (mo)</th>
<th>Transfusions</th>
<th>Abdominal Pain/Thromboembolism</th>
<th>Follow-Up (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.P.</td>
<td>Negative</td>
<td>ATG/CsA</td>
<td>6</td>
<td>None</td>
<td>None</td>
<td>54</td>
</tr>
<tr>
<td>B.E.</td>
<td>Negative</td>
<td>ATG/CsA</td>
<td>3</td>
<td>PRBC</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>B.H.</td>
<td>Negative</td>
<td>ATG/CsA</td>
<td>18</td>
<td>None</td>
<td>None</td>
<td>30</td>
</tr>
<tr>
<td>S.S.</td>
<td>Negative</td>
<td>ATG/CsA</td>
<td>3</td>
<td>None</td>
<td>None</td>
<td>63</td>
</tr>
</tbody>
</table>

Abbreviation: PRBC, packed red blood cells.

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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 pCR1ITa 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 C3. This deletion resulted in a frameshift that generated a premature stop codon at 179. The second patient, B.H., showed a transition of C163 to T. This transition introduced an in-frame stop codon in place of Q55. 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 C55 to T, resulting in the replacement of R77 with a W, and the second, a transversion of T762 to A, introducing an in-frame stop codon (TAA) in place of Y762.

With respect to the patients with primary PNH, two patients, M.W. and S.T., showed single nucleotide deletions of A767 and C533, 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 578 and 579. 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 1 and the S' 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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Affected Neutrophils (%)</th>
<th>Mutation</th>
<th>Position</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA/PNH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.P.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>C39</td>
<td>Frame shift TGA778</td>
</tr>
<tr>
<td>B.E.</td>
<td>68</td>
<td>Exchange</td>
<td>C65, T, T762, A</td>
<td>R77 to W, Y294 to TAA</td>
</tr>
<tr>
<td>B.H.</td>
<td>14</td>
<td>Exchange</td>
<td>C163, T</td>
<td>Q55 to TGA</td>
</tr>
<tr>
<td>S.S.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>14 bp1143-1154</td>
<td>TGA782</td>
</tr>
<tr>
<td>PNH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.V.</td>
<td>&gt;95</td>
<td>Insertion</td>
<td>ATGT576/578</td>
<td>Frame shift TGA804</td>
</tr>
<tr>
<td>I.D.</td>
<td>&gt;95</td>
<td>Splice defect</td>
<td>GT-AT818/819</td>
<td>Truncated protein (292 aa)</td>
</tr>
<tr>
<td>M.W.</td>
<td>40</td>
<td>Deletion</td>
<td>A627</td>
<td>Frame shift TAA778</td>
</tr>
<tr>
<td>S.T.</td>
<td>&gt;95</td>
<td>Deletion</td>
<td>C675</td>
<td>Frame shift TAA822</td>
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</tbody>
</table>
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 (C59), and the other encompassed 14 nucleotides (T114 to T1154). 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 (A167 and C375), 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.3,13-18 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 remain asymptomatic. Tichelli et al 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 group 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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Genetic defects underlying paroxysmal nocturnal hemoglobinuria that arises out of aplastic anemia

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