Molecular Basis of the Heterogeneity of Expression of Glycosyl Phosphatidylinositol Anchored Proteins in Paroxysmal Nocturnal Hemoglobinuria

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The purpose of these studies was to determine the molecular basis of the phenotypic mosaicism that is a defining feature of paroxysmal nocturnal hemoglobinuria (PNH). Analysis of T cell clones from a female patient revealed four distinct phenotypes based on surface expression of glycosyl phosphatidylinositol-anchored proteins (GPI-AP). When PIG-A (the gene that is mutant in PNH) from these clones was analyzed, four discrete somatic mutations were identified. Analysis of X chromosomal inactivation among the abnormal T cell clones was consistent with polyclonality. Together, these studies demonstrate that the phenotypic mosaicism that is characteristic of PNH is a consequence of genotypic mosaicism and that, at least in this case, PNH is a polyclonal rather than a monoclonal disease. That four distinct somatic mutations were present in a single patient suggests that in conditions that predispose to PNH PIG-A may be hypermutable.

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PAROXYSMAL NOCTURNAL hemoglobinuria (PNH) is an acquired hemolytic anemia that is characterized clinically by recurrent episodes of intravascular hemolysis resulting in hemoglobinuria. The hemolysis is a consequence of the abnormal sensitivity of the erythrocytes to complement-mediated lysis. PNH erythrocytes are abnormally sensitive to complement because they are deficient in both decay accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (MIRL, CD59). In addition to these two complement regulatory proteins, PNH erythrocytes are deficient in a functionally diverse group of membrane proteins that share the common biochemical feature of being anchored to the cell by a glycosyl-phosphatidylinositol (GPI) moiety. Further, all hematopoietic elements (ie, erythrocytes, platelets, granulocytes, monocytes, and lymphocytes) are deficient in GPI-anchored proteins (GPI-AP). There is no evidence that PNH is inherited, and a familial pattern associated with the disease has not been observed. Together, these observations suggest that PNH arises as the result of a somatic mutation involving the totipotent hematopoietic stem cell and that the mutation affects a gene that is essential for the normal biosynthesis of the GPI anchor.

A gene that complements the abnormal expression of GPI-AP in PNH cell lines was identified by Takeda et al. The gene was designated PIG-A (for phosphatidylinositol glycans). A gene that is mutant in PNH encodes a protein that is essential for the normal synthesis of N-acetyl glucosaminyl-phosphatidylinositol, an early intermediate in the pathway of the GPI anchor synthesis. Additional studies by Takeda et al. showed that PNH cells harbored a somatic mutation in PIG-A. Those investigators also observed that a heterozygous mutation was sufficient to produce the PNH phenotype. The dominant expression of the somatic mutation was explained when PIG-A was mapped to chromosome Xp22.1. Since males have a single X chromosome, any functionally significant mutation in PIG-A is expressed. Females are functionally haploid due to X-inactivation. Thus, somatic mutations in PIG-A appear dominant when they occur on the active X chromosome. Collectively, these studies defined both the biochemical and the molecular bases of the deficiency of GPI-AP in PNH. Somatic mutations in PIG-A have been found in all cases of PNH reported to date.

A characteristic feature of PNH is the presence of circulating hematopoietic cells with variable expression of GPI-AP. In some patients, cells that are completely deficient in GPI-AP circulate along with cells that are partially deficient and with cells with normal GPI-AP expression. Two hypotheses that would explain this phenotypic mosaicism appeared plausible. According to the first hypothesis, a single abnormal clone arises as a result of a somatic mutation and gives rise to progeny with variable expression of GPI-AP. In this model, epigenetic factors must be invoked to account for the phenotypic heterogeneity. According to the second hypothesis, more than one somatic mutation occurs. In this model, more than one abnormal clone is present, and the progeny of each clone is phenotypically different because the genotype is different. To investigate the validity of these two hypotheses, studies were undertaken to characterize the relationship between PNH phenotype and PIG-A genotype in a patient with heterogeneous expression of GPI-AP.

MATERIALS AND METHODS

Cells. After obtaining informed consent, peripheral blood from the patient with PNH, from the parents of the patient, and from healthy volunteers was obtained by venepuncture. Erythrocytes, mononuclear cells (MNC), and polymorphonuclear leukocytes (PMN) were isolated using standard methods. Elutriation was used to isolate monocytes and lymphocytes from the PNH MNC pool. HeLa cells were maintained in culture using RPMI supplemented with 10% fetal calf serum (FCS).
FACS. DAF (CD55) and MIRL (CD59) expression on normal and PNH erythrocytes, PMN, MNC, and cultured T cells was analyzed by FACS using published methods. Before analysis, Fcγ receptor function on PMN, MNC, and cultured T cells was blocked by incubating cells at 4°C with 200 μg/mL of nonimmune rabbit IgG. Monoclonal anti-CD55 (1H4) was a gift from Dr Wendell F. Rossé (Duke University Medical Center, Durham, NC) and monoclonal anti-CD59 (IF5) was a gift from Dr Hidechika Okada (Nagoya City University, Nagoya, Japan). A minimum of five thousand gated events per sample were analyzed using a FACSCan (Becton Dickinson, Research Triangle Park, NC).

T lymphocytes. Peripheral blood MNC from normal volunteers and from the patient with PNH were isolated by density gradient centrifugation, and antigen-nonspecific T cells were developed according to published methods. In some instances, a portion of the cells was used to analyze DAF (CD55) and MIRL (CD59) expression by FACS (see above) or to isolate total RNA or DNA (see below).

**Enrichment of GPI-AP deficient T cells.** Normal and PNH T cells were resuspended to 4 × 10^6/mL in serum-free RPMI. Three 1 mL aliquots were centrifuged (10 minutes at 450g at room temperature), and the supernate was aspirated. Next, 500 μL of phosphate buffered saline (PBS) containing 100 μg/mL of monoclonal anti-MIRL and 500 μL of PBS containing a 1:500 dilution of monoclonal anti-DAF ascites were added, and the mixture was incubated on ice. After 30 minutes, the cells were washed twice in RPMI and resuspended in 1 mL. Next, 1 mL of PBS containing 4 × 10^6 paramagnetic, polystyrene beads bearing affinity-purified sheep anti-mouse IgG (Dynabeads M-450; Dynal International, Oslo, Norway) was added, and the samples were incubated on ice. After 45 minutes, a magnet was applied to the test tube containing the cell suspension, and subsequently, the supernate was recovered. The cells were washed twice with serum-free RPMI and resuspended to 1 × 10^7/mL in RPMI containing 10% pooled human AB serum. Aliquots of 1 mL were added to 24-well flat bottom plates. Next, 1 mL of RPMI containing 10% serum, 100 U of IL-2, 10 μg of PHA, and 5 × 10^4 irradiated, allogeneic MNC were added to each well. The cells were expanded in culture, and subsequently analyzed for DAF and MIRL expression by using FACS.

**Cloning of PNH T cells.** The negatively selected PNH T cells were isolated by density gradient centrifugation and resuspended to 10 cells/mL in RPMI containing 10% serum. Aliquots of 100 μL were pipetted into the wells of 96-well round bottom trays, and 100 μL of complete media containing 5 × 10^6 allogeneic feeder cells were added to each well. The procedure of replacing spent media and feeder cells was performed weekly as described above until each well contained 5 × 10^6 to 1 × 10^7 cells (usually at 1 to 2 weeks). Viable cells were recovered following density gradient centrifugation.

Total cellular RNA was extracted from the T cell clones using the guanidinium isothiocyanate/phenol chloroform single step. The RNA Isolation Kit purchased from Stratagene Cloning Systems (La Jolla, CA) was used for this procedure. Alternatively, the cells were lysed in 4 mol/L guanidinium buffer, and total RNA was isolated using a cesium chloride centrifugation method. DNA was purified using standard methods. The same methods were used to extract RNA and DNA from cultured T cells and from peripheral blood MNC and PMN. In all cases, 5 × 10^5 to 1 × 10^6 cells were used to prepare the RNA and 1 × 10^5 cells were used to prepare DNA.

**Cloning and sequencing of PIG-A cDNA by using reverse transcription and polymerase chain reaction (RT-PCR).** Two methods were used. Both of the methods have been described in detail elsewhere.

**Direct sequencing of genomic DNA.** Six oligonucleotide primer sets (A-PR1 and B-PR1; A-IT1 and B-1; A-1 and B-IT1; A-IT2 and B-IT2; A-IT2 and B-IT3; A-IT4 and B-12) described by Fida et al were used to amplify regions 1 through 6 of PIG-A. These regions included 583 bp of 5' flanking sequence containing the PIG-A promoter and exon 1, the complete coding sequence contained in exons 2 to 6, and the intron-exon boundaries of these regions. The PCR products were used as templates for direct sequencing by dyeoxy chain termination using either the Taq Dye Primer Cycle Sequencing Kit or the Taq Dye Terminator Cycle Sequencing Kit and a Model 373A DNA Sequencer (Applied Biosystems, Division of Perkin-Elmer, Foster City, CA).

**Cloning and sequencing of PIG-A genomic DNA.** PCR primers that flanked the region of interest were used to amplify DNA from T cell clone 2D8. The sense primer was A-PR1 and the antisense primer was 5'-CAGAATCTCTAGCTTAAAGC. The antisense primer was synthesized based on sequence data obtained from a genomic clone that contains part of intron 1. This clone was kindly provided by Dr Taroh Kinoshita (Osaka University, Osaka, Japan). The mutant PCR product was cloned directly by using the TA Cloning Kit (Invitrogen Corp., San Diego, CA) and subsequently sequenced by using the dyeoxy method.

**Restriction analysis of PCR products.** The primer sets that flanked the appropriate regions of PIG-A cDNA or genomic DNA were used to generate PCR products that included the mutations observed in the T cell clones. The samples were incubated with the appropriate restriction enzymes (either Acc I or Spe I; New England Biolabs, Inc, Beverly, MA) and analyzed by electrophoresis on agarose gels followed by ethidium bromide staining.

**Northern analysis.** The experiments were performed according to the method of Ware, Rosse, and Howard. The oligonucleotide probe was developed from the full length PIG-A cDNA described by Miyata et al that was kindly provided by Dr Taroh Kinoshita (Osaka University). For use in Northern analysis, the full length cDNA was digested with Nco I (New England Biolabs, Inc) as described by Bessler et al, to produce a 2683 bp probe. This Nco I/Nco fragment consists of PIG-A nucleotides −79 to 2606.

**Southern analysis.** Aliquots of 10 μg of isolated DNA were digested with either BglII or BstII and Sac I and electrophoresed in a 0.8% agarose gel. Southern analysis was performed using standard methods. The BglII blot was probed with the Nco I/Nco fragment described above. The BstII/Sac I blot was analyzed using a 662 bp PIG-A probe consisting of nucleotides −583 to 23 along with the first 55 nucleotides of the 5' end on intron 1. This probe followed the promoter probe since it contained the PIG-A promoter
erythrocytes were analyzed for expression of the two GPI-APs. The patient with PNH demonstrated heterogeneity of expression of the GPI-APs. Normal erythrocytes showed uniform, positive staining with both anti-MIRL and anti-DAF (Fig 1). In contrast, analysis of MIRL and DAF on the erythrocytes of a patient with PNH demonstrated heterogeneity of expression (Fig 1). When monoclonal anti-DAF was used as the primary antibody, ~20% of the circulating erythrocytes showed negative expression, ~75% showed intermediate expression, and ~5% showed normal expression (Fig 1). When monoclonal anti-DAF was used as the primary antibody, the FACS analysis was also abnormal, but the division into three discrete populations was less obvious (Fig 1).

Brief case history. The cells used in this study were obtained from a 35-year-old woman who was diagnosed with aplastic anemia in 1979 at the age of 19. In 1982, she was treated with high dose, pulse methylprednisolone. One year later the disease ameliorated, and she became transfusion independent. In 1985 she experienced an episode of hemoglobinuria, and the diagnosis of PNH was subsequently established based on a positive acidified serum lysis test.

Development of PNH clones with variable expression of GPI-AP. To investigate the relationship between PIG-A genotype and PNH phenotype, T cell clones with variable expression of GPI-AP were developed. FACS analysis of the clones showed four distinct phenotypes (Fig 2A and B). The phenotype represented by clones 2M6 and 2D10 was uniformly positive for both DAF and MIRL expression. Accordingly, this phenotype was designated (+ +). Clones 2M23 and 2D1 have similar phenotypes, with slightly discordant expression of DAF and MIRL. DAF expression by these clones is borderline positive, and MIRL expression is primarily negative. Thus, the phenotype of these clones was designated (+ -). Clones 2D36 and 1M9 show concordant, borderline positive expression of both DAF and MIRL; this phenotype was designated (± ±). Another clone, 2M10, with this phenotype was identified and included in some of the subsequent analyses. Expression of both DAF and MIRL falls within the negative range for clones 2D13 and 2D39; therefore, the phenotype represented by these was designated (- -). Two additional clones, 2D5 and 2D8, with the (- -) phenotype were identified and included in some of the studies described below. We have repeatedly analyzed DAF and MIRL expression by these clones over a period of over 1 year, and changes in phenotype have not been observed. During this period of observation, aliquots of the clones have been frozen and subsequently re-expanded, and phenotypic fidelity has been maintained.

T cells from normal volunteers were also subjected to negative selection using anti-DAF and anti-MIRL antibodies. In some cases, the negative selection was repeated on the cells after they had been re-expanded following the initial negative selection. Cell sorting was also used in an attempt to develop GPI-AP deficient T cells from normal donors. Using these techniques, we have been unable to identify GPI-AP deficient T cells in the peripheral blood of normal volunteers. In addition, we established T cell clones following the negative selection procedures. Although the exact number of individual T cell clones that have been analyzed...
Fig 2. (A and B) FACS analysis of PNH T cell clones. Negatively selected PNH cells were cloned by limiting dilution, and expression of GPI-AP was analyzed by FACS using anti-DAF (CD55) and anti-MIRL (CD59) as primary antibodies. Clones with four distinct phenotypes were observed.

for GPI-AP expression was not determined, 20 to 30 is a reasonable estimate. Uniformly, those clones showed normal GPI-AP expression. It seemed counterproductive to screen larger numbers of T cell clones derived from normal volunteers for GPI-AP expression without some evidence that a deficient population was present after the negative selection. These experiments suggest that a subpopulation of GPI-AP deficient stem cells is not a component of normal bone marrow.

Northern analysis of PIG-A expression by PNH T cell clones. Miyata et al\textsuperscript{23} have observed a single 4.2 kb transcript when total cellular RNA was analyzed using a PIG-A specific oligonucleotide probe. A prominent band of approximately 4.0 kb was visible in the lanes containing total RNA from the clones with normal GPI-AP expression (2M6 and 2D10, Fig 3). PIG-A mRNA expression by clone 1M9 (± phenotype) was similar to that of 2M6 and 2D10 (Fig 3), suggesting that the abnormal phenotype was due to a mutation that affected the functional activity of the PIG-A protein. In contrast, PIG-A transcripts were reduced (2D1 and 2M23) or undetectable (2D13) in other T lymphocyte clones, suggesting that these cells harbored mutations that affected PIG-A transcription or mRNA stability.

Molecular basis of the abnormal expression of GPI-AP by the PNH T-cell clones. Sequencing of PIG-A from the two clones with normal GPI-AP expression (2M6 and 2D10, Fig 2) revealed no somatic mutations. In contrast, four discrete mutations in PIG-A were observed among the clones with abnormal phenotypes.

Clone 2M23. By using RT-PCR, PIG-A cDNA from 2M23 was cloned and sequenced. All clones analyzed showed a single base (thymine) deletion at position 564. This deletion causes a frame shift after codon 187 and introduces a premature stop signal at codon 193. Inasmuch as the full length PIG-A protein consists of 484 amino acids,\textsuperscript{22} the predicted mass of the truncated protein product is 40% of normal. This single base deletion also influences PIG-A mRNA expression (Fig 3). Together, these results suggest a plausible explanation for the abnormal GPI-AP expression by clone 2M23 since the mutation appears to affect PIG-A both quantitatively and functionally.

The deletion at position 564 eliminates an Spe I restriction site. We took advantage of this modification to characterize further the mutation in clone 2M23. PCR primers\textsuperscript{11} that flank the site of the deletion were used to amplify a 629 bp product, and cDNA prepared from clones 2M6 and 2M23, from HeLa cells [the cell line from which PIG-A was originally cloned\textsuperscript{23}], and from peripheral blood PMN from the patient and her parents was analyzed. After incubation with Spe I, the PCR products from the HeLa cells and 2M6 were com-
Fig 3. Northern analysis of representative PNH T cell clones. Upper panel: Aliquots of 8 μg of total cellular RNA were analyzed. The source of the RNA is shown above the lanes along with the phenotype of the PNH T cell clones. Left, the positions of the size standards in kilobases (kb) are indicated. Lower panel: After removing the radioactivity, the blot was analyzed using a glyceraldehyde phosphate dehydrogenase (GPDH) probe in order to confirm the integrity of the RNA and demonstrate that each lane contained approximately the same amount of RNA. Expression of PIG-A mRNA by the clones with abnormal phenotypes was variable.

Fig 4. Spe I restriction analysis. (A) Analysis of RT-PCR products. cDNA was prepared from PNH T cell clones 2M6 (+ + phenotype) and 2M23 (± – phenotype), from HeLa cells, and from the peripheral blood PMN of the patient’s father (Fa), the patient’s mother (Mo), and the patient (PNH). A primer set that flanks the site of the mutation in 2M23 was used to amplify a 629 bp segment of PIG-A cDNA. The PCR products were incubated with Spe I, and subsequently analyzed by agarose gel electrophoresis and ethidium bromide staining. Spe I cleaves the normal PCR product into two fragments (521 bp and 108 bp). The mutation in clone 2M23 eliminates the Spe I restriction site. Left, the size of the stained bands is indicated. (B) Analysis of genomic PCR products. DNA was prepared from circulating PMN from a normal volunteer donor (NL PMN) and the patient with PNH (PNH PMN), and from PNH T cell clones 2D10 (+ + phenotype), 2M23 (± – phenotype), 2D1 (± – phenotype), 2D5 (– – phenotype), and 2D36 (+ + phenotype). A primer set that flanks the site of the mutation in 2M23 was used to amplify a 497 bp segment of PIG-A. The PCR products were incubated with buffer (–) or with buffer containing Spe I (+), and subsequently analyzed by agarose gel electrophoresis and ethidium bromide staining. Spe I cleaves the normal PCR product into two fragments of 256 bp and 241 bp that are not resolved by the gel and thus appear as a single band (marked 249 bp). The mutation in clone 2M23 eliminates the Spe I restriction site. The PCR product from 2M23 was partially digested, indicating that PIG-A was mutated on one of the X chromosomes. Left, the size of the stained bands are indicated. The lane marked St contains a set of size standards generated by HindII digestion of λ DNA. The size of the most inferior band in that lane is 564 bp.
nated PNH in Fig 4A), two bands were observed. The less intense band represents undigested cDNA, indicating that a small proportion of the mRNA from circulating PMN harbor the same mutation as 2M23. This finding indicates that the mutation is present in a primitive stem cell that gives rise to granulocytes as well as lymphocytes, and supports the conclusion that the mutation is not an artifact that arose as a consequence of the T cell cloning process.

Experiments designed to determine if the mutation in the cDNA prepared from 2M23 was also present in the genomic DNA were performed. DNA from normal and PNH peripheral blood PMN, and from PNH T cell clones 2D10 (± +, Fig 2B), 2M23 (± −, Fig 2A), 2D1 (± −, Fig 2B), 2D5 (− −, not shown), and 2D36 (± ±, Fig 2A) was isolated, and PCR primers that flank the site of the deletion were used to amplify a 497 bp segment of PIG-A. Incubation of the PCR product from normal PMN with Spe I showed complete cleavage, whereas a portion of the DNA from PNH PMN was undigested (Fig 4B). These results demonstrate that some of the circulating PMN from the patient harbor the mutant PIG-A allele.

There was complete cleavage of the genomic PCR products from all of the T cell clones except 2M23, which showed partial cleavage (Fig 4B). These findings confirm that genomic DNA from 2M23 contains the same mutation as the cDNA. Direct sequencing of the genomic DNA from 2M23 was consistent with a heterozygous deletion at position 564 (not shown). That only partial cleavage of the PCR product was observed is consistent with the interpretation that only one of the PIG-A alleles of 2M23 is mutated, while the complete digestion by Spe I of the PCR products derived from the other PNH clones demonstrates that the mutation is not inherited. Together, these results confirm the somatic origin of this mutation. The phenotype of clone 2D1 is similar to that of 2M23 (Fig 2, A and B), but complete cleavage of 2D1 by the restriction enzyme demonstrates that the genotypic basis for the abnormal phenotype is different. Using this same rationale, different genotypic abnormalities must also account for the abnormal phenotypes of clones 2D5 and 2D36.

Clone 2D1. As discussed above, the phenotype of 2D1 is similar to that of 2M23 (± −, Fig 2, A and B), but based on restriction analysis (Fig 4B), the genotypes are different. Northern analysis demonstrated that PIG-A mRNA expression by clone 2D1 was reduced (Fig 3). Sequencing of PIG-A cDNA from 2D1 showed a single nucleotide (G) deletion at position 849. The deletion involved a triplet mononucleotide repeat (GOG). The most 5' G of this triplet is the most 3' nucleotide of exon 3, while the other two Gs of the triplet are the most 5' nucleotides of exon 4. Direct genomic sequencing of exon 3 and its intronic boundaries was normal. In contrast, direct genomic sequencing of the boundary between intron 3 and exon 4 showed a heterozygous G to A substitution involving the acceptor splice site [the wild type cagGGT was changed to caaGGT (intronic sequence shown in lower case letters and exonic sequence shown in upper case letters, with the mutated site shown in bold letters)]. This mutation explained the G deletion observed in the cDNA from 2D1 since it caused the acceptor splice site (AG) to be shifted by 1 nucleotide. While no aberrant splicing of PIG-A was observed by RT-PCR analysis, the mutation caused a frame shift after codon 283 and introduced a premature stop signal at codon 290. The effects of the mutation on both PIG-A mRNA expression (Fig 3), and PIG-A structure provide an explanation for the GPI-AP deficiency of 2D1 (Fig 2B).

Clones 2D36 and 1M9. To establish the molecular basis of the abnormal phenotype of PNH T cell clone 2D36, the PIG-A cDNA was sequenced. A single base substitution (the wild type adenine is changed to cytosine) at position 304 was observed. As a consequence, threonine at codon 102 is changed to proline. Presumably, this substitution affects the functional activity of the PIG-A protein. Support for this conclusion is provided by the observation that the threonine at this position is conserved in both murine PIG-A and SPT-14 from Saccharomyces cerevisiae, the putative yeast homolog of PIG-A. Apparently, however, this amino acid substitution only partially inactivates the PIG-A protein since some GPI-AP expression by 2D36 is observed (Fig 2A). Additionally, this mutation appears to have no effect on PIG-A mRNA expression (Fig 3).

The missense mutation at position 304 eliminates an Acc I restriction site. RT-PCR primers that flank the site of the mutation were used to amplify a 597 bp product. The PCR product from 2D36 that was incubated with Acc I was undigested (Fig 5A), confirming that the mutated PIG-A was on the active X chromosome. Identical results were observed for the RT-PCR product derived from 2M10 (Fig 5A), a PNH T cell clone with the same phenotype (± ±, not shown) as 2D36. In contrast, incubation of PIG-A cDNA from 2M6 (+ +, phenotype, Fig 2A) with Acc I showed the expected bands of 368 and 229 bp, indicating that this clone was not mutated at position 304. Restriction analysis of the PIG-A cDNA from PNH peripheral blood PMN showed that the majority of the PCR product was mutated (Fig 5A). These findings indicate that most of the PIG-A mRNA from the circulating PMN is derived from a primitive stem cell that harbors the mutation at position 304. Faint bands at 368 and 229, however, imply that a small portion of the PNH PMN mRNA arises from stems cells that are not mutated at position 304.

To confirm that the genomic DNA harbored the same mutation as the cDNA, primers that flanked the site of the deletion were used to amplify a 487 bp product. Following incubation with Acc I, the PCR product derived from PNH T cell clone 1M9, that has the same phenotype as 2D36 (± ±, Fig 2, A and B) showed that this abnormal clone harbors the same mutation (Fig 5B). That only part of the PCR product was undigested is consistent with the interpretation that only one of the PIG-A alleles was mutated. In support of this conclusion, heterozygosity at position 304 was confirmed by direct sequencing of the genomic DNA from clone 1M9 (not shown). The somatic origin of this mutation was demonstrated by showing that genomic DNA from the patient’s parents was not mutated at position 304 since the PCR product derived from their DNA was completely digested by Acc I (Fig 5B). This conclusion was further substantiated by experiments that revealed that the
genomic PCR product from two other PNH T cell clones with different phenotypes (2M23 and 2D39, Fig 2, A and B) was also completely digested with Acc I (not shown). Additional studies confirmed that the PIG-A mutation affected a primitive hematopoietic progenitor that gives rise to monocytes and lymphocytes as well as PMN (Fig 5B). Further, this stem cell appears to be clonally dominant. This conclusion is based on the observation that, in the samples derived from the PNH lymphocytes, monocytes, and PMN, the intensity of the 487 bp band is approximately equal to that of the 393 bp band (Fig 5B), indicating that the majority of the circulating cells contained the mutant allele.

Clones 2D13 and 2D39. PNH T cell clones 2D13, 2D39, and 2D8 have the same phenotype (+ +, Fig 2, A and B, and not shown, respectively), and Northern analysis showed markedly reduced expression of PIG-A mRNA by these clones (Fig 6A). To investigate the hypothesis that a relatively large deletional mutation accounted for the abnormal PIG-A, genomic DNA from the clones with the (+ +) phenotype was analyzed by Southern blot. The lanes containing DNA from clones 2D8, 2D13, and 2D39 (lanes 3, 4, and 5, Fig 6B) showed a band that was not seen in the lanes containing the DNA from the other sources. The absence of this additional band from the lanes containing the DNA from clone 2D1 and from the PMN of the patient’s mother and father (Fig 6B) demonstrate that the aberrant band was not the result of an inherited polymorphism, but rather arose as a consequence of a somatic mutation of PIG-A. Further, that the four normal bands were also present in the lanes containing DNA from clones 2D8, 2D13, and 2D39 indicates that the mutation affected only one PIG-A allele. Subsequent Southern analysis showed that another PNH T cell clone (2D5) with the same phenotype as 2D8, 2D13, and 2D39 had the same genotypic abnormality (not shown).

The deletional mutation was characterized further using additional oligonucleotide probes and more extensive digestion of DNA using a combination of BglII and Sac I. Together, those studies demonstrated that the abnormal band seen in the lanes containing DNA from clones 2D8, 2D13, and 2D39 (Fig 6B) results from a deletion involving a BglII/Sac I fragment of PIG-A that includes a portion of the promoter region, exon 1, and the exon 1-intron 1 boundary (data not shown). Based on this information, oligonucleotide primers that flanked the deletion were used to amplify genomic DNA from clone 2D8. Sequencing of the mutant PCR product revealed a 737 bp deletion that included part of the promoter region, all of exon 1, and 577 bp of the 5’ end of intron 1 (Fig 6C). Presumably, this deletion accounts for the absence of PIG-A mRNA and ultimately for the abnormal phenotype of clones 2D8, 2D13, 2D39, and 2D5. A summary of the circulating cells contained the mutant allele.

Additional genomic PCR product from two other PNH T cell clones with different phenotypes (2M23 and 2D39, Fig 2, A and B) was also completely digested with Acc I (not shown). Additional studies confirmed that the PIG-A mutation affected a primitive hematopoietic progenitor that gives rise to monocytes and lymphocytes as well as PMN (Fig 5B). Further, this stem cell appears to be clonally dominant. This conclusion is based on the observation that, in the samples derived from the PNH lymphocytes, monocytes, and PMN, the intensity of the 487 bp band is approximately equal to that of the 393 bp band (Fig 5B), indicating that the majority of the circulating cells contained the mutant allele.

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Fig 6. Analysis of clones with the (− −) phenotype. (A) Northern analysis. Upper panel: Aliquots of 15 µg of total cellular RNA were analyzed. The source of the RNA is shown above the lanes along with the phenotype of the PNH T cell clones. NL, RNA derived from cultured T cells from a normal volunteer. Left, the positions of the size standards in kilobases (kb) are indicated. Lower panel: After removing the radioactivity, the blot was analyzed using a GPDH probe in order to confirm the integrity of the RNA and demonstrate that each lane contained approximately the same amount of RNA. Expression of PIG-A mRNA by the all three clones with the (− −) phenotype was markedly reduced. (B) Southern analysis of Bgl II digested DNA. Aliquots of 10 µg of DNA were analyzed. The sources of the DNA were as follows: PMN from a normal female donor, lane 1; 2D1 (± − phenotype), lane 2; 2D8 (− − phenotype), lane 3; 2D13 (− − phenotype), lane 4; 2D39 (− − phenotype), lane 5; PMN from the patient’s mother, lane 6; PMN from the patient’s father, lane 7; PMN from a normal male donor, lane 8. The band at 6.6 kb is derived from the PIG-A pseudogene. The other bands represent fragments of PIG-A. An additional band is present in the lanes containing the PNH T cell clones with the (− −) phenotype. This band is not seen in the DNA from either the other T cell clone or the patient’s parents, demonstrating the somatic origin of the mutation. Left, the positions of the size markers are indicated. (C) Schematic representation of the PIG-A deletion affecting clones with the (− −) phenotype. PCR primers (indicated by the closed rectangles above and below the horizontal line) that flanked the deletion were used to amplify genomic DNA from clone 2D8. Sequence analysis of the mutant PCR product showed a 737 bp deletion (denoted by the open rectangle). The positions of the putative cis-acting elements are indicated by the vertical lines based on the report of Iida et al. 77 T cell clones with this deletion express little or no PIG-A mRNA.

of the analysis of the abnormal T cell clones is shown in Table 1.

Determination of clonality by analysis of X chromosomal inactivation. Studies by other investigators have suggested that PNH is a monoclonal disease.18,19 If this interpretation is correct, all of the abnormal PNH T cell clones identified in the present study would have to arise from a common ancestor. To investigate this hypothesis, X chromosomal inactivation among the various PNH T clones was determined. In these experiments, genomic DNA is incubated

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with buffer or with buffer containing the methylation-sensitive restriction enzyme Hpa II. Next, a highly polymorphic region of the human androgen receptor gene (HUMARA) that is located on the X chromosome is amplified by PCR. The PCR primers flank two Hpa II sites near the polymorphic region of HUMARA. Thus, only the inactive (methylated) gene is amplified following treatment with HpaII since the restriction enzyme disrupts the continuity of the sequence between the PCR primers on the active (unmethylated) gene.

In the buffer control samples [the (−) samples] from females, both the active and the inactive genes are amplified. When analyzed using denaturing polyacrylamide gel electrophoresis, four major bands are seen because both the sense and the antisense strands from the PCR products derived from both the maternal and the paternal gene are resolved (Fig 7). In the HpaII treated samples [the (+) samples] from females, polyclonality is defined by the appearance of all four bands, indicating random X inactivation among the cells from which the DNA was derived. In contrast, monoclonality is defined by nonrandom X inactivation. In this case, only two bands (from either the maternal or the paternal PCR product) are seen in the samples treated with HpaII.

Analysis of DNA from the peripheral blood PMN of the patient’s mother was informative since the maternal and paternal genes were sufficiently polymorphic to allow resolution. The appearance of four bands in the HpaII-treated sample indicated polyclonality (Fig 7). As evidenced by the equivalent intensity of both sets of bands, the two alleles appeared to be represented equally. This finding is consistent with random X inactivation without skewed X-inactivation. As anticipated, analysis of the DNA from the patient’s father showed evidence of only 1 X chromosome, and that X chromosome must be unmethylated (ie, active) since no band was seen in the HpaII-treated sample (Fig 7). Analysis of DNA from the peripheral blood PMN from the patient was informative. The easily discernable difference in the electrophoretic mobility of the PCR products between the maternal and paternal samples clearly defines the derivation of the patient’s two alleles. The upper set of bands is derived from the maternal allele, whereas the lower set is derived from the paternal allele. The presence of both sets of bands in the HpaII-treated sample demonstrates polyclonality; however, the upper set of bands is more intense than the lower set. This finding could result from skewed X-inactivation or from dominance of the totipotent hematopoietic stem cell pool by a population of cells in which the paternal X chromosome is active. Analysis of X chromosomal inactivation in other somatic tissues is required to distinguish between these two interpretations.

The pattern of X chromosomal inactivation in the PNH PMN is consistent with polyclonality; however, since there appears to be a small population of peripheral blood cells with normal GPI-AP expression (Fig 1), the residual normal stem cells could account for the polyclonality, and the abnormal cells could still be monoclonal. To investigate this hypothesis, the pattern of X chromosomal inactivation among each of the PNH T cell clones was determined. All nine samples gave the expected nonrandom pattern of X inactivation, confirming that in each case the limiting dilutional procedure lead to the cloning of a single T lymphocyte. Seven clones showed inactivation of the maternal X chromosome while two showed inactivation of the paternal X chromosome (Fig 7). Inasmuch as they have the same genotypic abnormality, clones 1M9 and 2D36 (Fig 5B) must be derived from a common ancestor. Using this same rationale, 2D8, 2D13, and 2D39 belong to the same clone. Thus, three abnormal clones (represented by 2M23, 2D1, and the group of 2D8, 2D13, and 2D39) have an inactive maternal X chromosome, while one of the abnormal clones (represented by 1M9 and 2D36) has an inactive paternal X chromosome. Thus, all of the T cell clones with mutant PIG-A could not have arisen from a single common ancestor since the inactive X allele is not the same for all of the clones. These results demonstrate that, at least in this case, PNH is a polyclonal rather than a monoclonal disease. Further support for this conclusion is provided by the observation that since four separate PIG-A mutations are present, the mutational events must have occurred independently rather than by clonal evolution.

### DISCUSSION

The purpose of these studies was to determine the molecular basis of the heterogeneity of expression of GPI-AP in patients with PNH. From a single patient, T cell clones with three distinct abnormal phenotypes were developed. Among these clones, four separate mutations that affect the function of PIG-A were identified. Together, these studies demonstrate that genotypic mosaicism accounts for the phenotypic mosaicism characteristic of PNH.

Among patients with PNH, the percentage of peripheral

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**Table 1. Summary of the PIG-A Mutations in the T Cell Clones From a Patient With PNH**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype*</th>
<th>mRNA Expression†</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M23</td>
<td>(± –)</td>
<td>Partially reduced</td>
<td>T deletion at position 5641 causes a frameshift and introduces a premature stop codon.</td>
</tr>
<tr>
<td>2D1</td>
<td>(± –)</td>
<td>Partially reduced</td>
<td>G to A substitution involving the acceptor splice site at the boundary of intron 3 and exon 4. Splice site shifted by 1 base, causing deletion of the first nucleotide of exon 4. The resulting frameshift introduces a premature stop codon.</td>
</tr>
<tr>
<td>2D38, 1M9</td>
<td>(± ±)</td>
<td>Normal</td>
<td>A to C substitution at position 304. Threonine at codon 102 changed to proline.</td>
</tr>
<tr>
<td>2D13, 2D39</td>
<td>(– –)</td>
<td>Markedly reduced</td>
<td>737 bp deletion involving the 5′ untranslated (promoter) region, exon I, and intron I.</td>
</tr>
</tbody>
</table>

* The phenotype is based on FACS analysis of the expression of the GPI-AP complement regulatory proteins DAF (CD55) and MlRL (CD59), respectively (Fig 2, A and B): ±, partial expression; −, minimal or no expression.

† Northern analysis of PIG-A mRNA expression is shown in Figs 3 and 8A.

‡ The nucleotides are numbered starting with the first nucleotide of the coding region of PIG-A.
blood cells that are GPI-AP deficient varies widely. Thus, some patients with PNH may have <5% of their circulating cells that are GPI-AP deficient, whereas others may have >90% abnormal cells. Our studies suggest that the proportion of active stem cells that are mutated will impact on the percentage of GPI-AP deficient cells in the peripheral blood. The patient studied herein had at least four stem cells with functionally significant mutations in PIG-A. The percentage of the active stem cell pool that is abnormal is unknown since the total number of active stem cells (ie, normal + abnormal) is indeterminant. Based on the FACS analysis, however, it seems likely that the normal component of the active stem cell pool is relatively small since only 5% of the PNH erythrocytes have normal GPI-AP expression (Fig 1). Thus, in this case, the majority of active stem cells may be mutant. Inasmuch as PNH appears to have evolved from aplastic anemia in this patient's case, her total pool of hematopoietic progenitors may be abnormally low. Therefore, in other cases in which the percentage of affected peripheral blood cells is lower, the absolute number of normal stem cells may be higher, or fewer of the active stem cells may be mutant (or both).

Another important factor that appears to contribute to the phenotypic pattern of the GPI-AP deficient cells in the peripheral blood is the proliferative capacity of the affected stem cell. For example, the phenotype of the patient's erythrocytes (Fig 1) appears to be due in part to clonal dominance by the stem cell that gives rise to progeny that are partially deficient in GPI-AP (represented by clones 2D36 and 1M9, Fig 2), since the majority of circulating cells contain the same PIG-A mutation as those clones (Fig 5A and B). Currently, however, there is no evidence that a particular GPI-AP phenotype or PIG-A genotype per se influences proliferative capacity. Rather, it seems more likely that clonal dominance is determined by the intrinsic growth properties of the affected stem cell.

Our studies indicate that different mutations in PIG-A can produce subtle but discrete differences in GPI-AP expression (Fig 2A and B and Table 1). Thus, clones with the (-) phenotype have a different genotype compared with those with the (± -) phenotype, which in turn have a PIG-A genotype that is distinct from clones with the (± ±) phenotype. Based on these observations, we conclude that PIG-A genotype determines PNH phenotype. Cells with the (± ±) phenotype (represented by clone 1M9) appear to have comparatively normal PIG-A mRNA expression (Fig 3). In this case, the deficient GPI-AP protein expression is almost certainly a consequence of a missense mutation that alters a highly conserved amino acid. Despite the mutation, the PIG-A protein must retain some function since clones with this defect are only partially deficient in GPI-AP. The erythrocyte progeny of abnormal stem cells with this type of mutation are classified as PNH type II since they have partial expression of DAF (CD55) and MIIR (CD59) (Fig 1) and are therefore more resistant to complement-mediated lysis than the PNH type III cells that are completely deficient in the GPI-anchored complement regulatory proteins.

The present studies also show that similar phenotypes can arise from distinct genotypic abnormalities. This conclusion is based on the observation that clones 2M23 and 2D1 have phenotypes that are indistinct (Fig 2, A and B) but that the PIG-A mutations that account for the abnormal GPI-AP expression are discrete (Table 1). That 2M23 and 2D1 have partial (albeit very low) GPI-AP expression (Fig 2, A and B) is surprising since the mutations should result ultimately in the synthesis of a PIG-A protein in which 50% to 60% of the carboxy-terminus is absent. Nonetheless, that GPI-AP expression is observed on 2M23 and 2D1 cells indicates that even these severely truncated proteins retain some functional activity.

Others have also found more than one type of PIG-A mutation in cells from individual patients with PNH. Bessler et al developed EBV-transformed B lymphocyte cell lines from patients with PNH and identified clones with abnormal GPI-AP expression by FACS analysis. In two individuals, two clones with different PIG-A mutations were observed. Apparently, the phenotype of all of those clones was the same [similar to the (--) phenotype in the present studies]. The authors speculated that the mutant clones expanded because the GPI-AP deficiency provided a selective advantage in the setting of abnormal hematopoiesis. While we agree that under some conditions GPI-AP deficiency may provide hematopoietic stem cells with a survival advantage, our studies suggest a hypothesis that is different from the proposed by Bessler et al. That we identified four separate somatic mutations in PIG-A affecting the peripheral blood cells of a single patient with an antecedent history of aplastic anemia implies that abnormal hematopoiesis may influence the mu-
tational rate of the gene. Our interpretation of these observations is that hypermutability of PIG-A in addition to convergent evolution accounts for the genotypic mosaicism of PNH. Additional support for the suggestion that PIG-A can be hypermutable is provided by the recent finding of Ostendorf et al of two separate somatic mutations involving the same PIG-A gene in a single patient with PNH. This finding also suggest the possibility that PIG-A is targeted for mutation.

Yamada et al analyzed the PIG-A from the peripheral blood PMN of 14 patients with PNH. They found one patient with two discrete PIG-A mutations. Based on those observations, one would conclude that genotypic mosaicism is relatively uncommon in PNH. It seems likely, however, that the method used by Yamada et al to characterize the PIG-A mutations in PNH underestimates both the number of patients with multiple abnormal clones and the number of abnormal clones per patient. Those investigators identified mutant transcripts by transfecting a complementation class A cell line with PIG-A cDNA prepared from the patients’ peripheral blood PMN. Samples that did not complement GPI-AP protein expression by the class A mutant were subsequently sequenced. This type of analysis will identify only those mutations that affect the coding region of PIG-A. Further, mutations that negatively affect mRNA expression are also likely to be missed since PIG-A transcripts from these abnormal cells will be under-represented. Thus, if the method of Yamada et al had been used to characterize the PIG-A mutations in our patient, it is likely that only the mutation represented by clone 2D36 would have been identified. Based on the observation that phenotypic mosaicism is common in PNH, it seems likely that genotypic mosaicism is also common and that the method used to detect PIG-A mutations will have a significant impact on the estimation of the frequency and extent of the mosaicism. Our analysis may also have left additional PIG-A mutations undiscovered since, as noted above, clones with similar phenotypes may be genotypically distinct.

Together, the studies presented herein suggest that the following three factors determine the PNH phenotype of the peripheral blood of a particular patient: (1) The type of mutation (ie, whether the mutation partially or completely destroys the functional activity of the PIG-A protein). Mutations that cause partial loss of function (ie, missense mutations) produce the PNH II phenotype, whereas mutations that cause complete loss of function (ie, deletions, insertions, or nonsense mutations) produce the PNH III phenotype. (2) The number of mutations (ie, the greater the number of mutant stem cells, the greater the proportion of GPI-AP deficient peripheral blood cells); (3) the proliferative properties of the affected stem cells (ie, involvement of clonally dominant stem cells has a greater impact on the phenotype than involvement of relatively quiescent stem cells). Additional studies are required to delineate the mechanism that underlies the genotypic mosaicism of PNH.

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Molecular basis of the heterogeneity of expression of glycosyl phosphatidylinositol anchored proteins in paroxysmal nocturnal hemoglobinuria

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