Syngeneic Bone Marrow Transplantation Without Conditioning in a Patient With Paroxysmal Nocturnal Hemoglobinuria: In Vivo Evidence That the Mutant Stem Cells Have a Survival Advantage

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A 10-year-old girl with paroxysmal nocturnal hemoglobinuria (PNH) received an infusion of syngeneic bone marrow without preparative marrow ablation or immunosuppression. Following transplant, the patient became asymptomatic in concordance with an increase in the percentage of peripheral blood cells with normal expression of glycosylphosphatidylinositol–anchored proteins (GPI-AP). However, molecular analysis suggested engraftment of a relatively small number of donor stem cells and persistence of an abnormal stem cell with mutant PIG-A. During 17 months of observation, the percentage of cells with normal GPI-AP expression gradually decreased, while intravascular hemolysis progressively increased. Approximately 16.5 months post-transplant, the patient once again became symptomatic. Together, these results indicate that syngeneic marrow infusion provided a clinical benefit by increasing the proportion of erythrocytes with normal expression of GPI-anchored complement regulatory proteins without supplanting the abnormal stem cells. However, evidence of insidious disease progression following the marrow infusion implies that the abnormal stem cells have a survival advantage relative to the transplanted stem cells. Thus, these studies contribute in vivo data in support of the hypothesis that PNH arises as a consequence of a pathological process that selects for hematopoietic stem cells that are GPI-AP–deficient.

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PAROXYSMAL NOCTURNAL hemoglobinuria (PNH) is an acquired disease in which a somatic mutation involving a primitive hematopoietic stem cell occurs. The gene that is mutated (PIG-A) is located on the X chromosome. PIG-A encodes a protein that is essential for the normal biosynthesis of the glycosylation phosphatidylinositol (GPI) moiety that serves as a membrane anchor for a functionally diverse group of cellular proteins.

The primary clinical manifestations of PNH are intravascular hemolysis resulting in hemoglobinuria, thrombosis, and abnormal hematopoiesis, which results in pancytopenia. The natural history varies greatly among patients. Depending on the disease manifestations, therapeutic options include supportive care, glucocorticoids, and anticoagulation. With particularly severe disease (eg, bone marrow failure or recurrent, life-threatening thromboembolic events), marrow ablation followed by bone marrow transplantation from an HLA-matched sibling donor should be considered. In this case, the abnormal stem cells are destroyed by the conditioning regimen, and the marrow is repopulated with normal donor cells. While this approach is potentially curative, the benefits must be weighed against the significant morbidity and mortality associated with allogeneic bone marrow transplantation.

In the unusual circumstance in which the patient has a syngeneic twin, bone marrow transplantation is the most appropriate therapy for severe PNH, since the absence of graft-versus-host disease greatly reduces transplant-associated morbidity and mortality. In four reported instances, patients with PNH have been infused with syngeneic marrow without pretransplant ablative or immunosuppressive therapy. Of these four cases, one patient showed no evidence of improvement or engraftment of donor cells. However, three patients had amelioration of symptoms. Of these three, one relapsed within 6 months and required a second transplant following conditioning with cyclophosphamide and total-body irradiation. Another was symptomatically improved more than 8 years after the transplant, although the basis of the clinical benefit is not apparent, as the peripheral blood of the patient contained 98% DAF (CD55)-negative cells.

The last patient had no evidence of PNH (based on negative sugar water and Ham’s tests) more than 17 years posttransplant (last reported in 1992).

We evaluated a 10-year-old patient with PNH who had an unaffected syngeneic twin. Because of debilitating symptoms, and because PNH with onset in childhood appears to have a worse prognosis than adult PNH, bone marrow transplantation was recommended. We elected to infuse syngeneic bone marrow in the absence of a conditioning regimen, because it is virtually free of complications and long-term improvement has been observed. If that therapy proved inadequate, the patient could be treated subsequently with a second syngeneic transplant after undergoing marrow ablative therapy. Here we report the effects of infusion of normal syngeneic marrow on the patient’s clinical course, on expression of GPI-anchored proteins (GPI-AP) in the peripheral blood, and on the contribution of the mutant stem cells to hematopoiesis.

MATERIALS AND METHODS

Cells. After informed consent, peripheral blood samples from the patient, her syngeneic twin, her mother, and healthy volunteers were obtained by venipuncture. Erythrocytes and polymorphonuclear leukocytes (PMN) were isolated using standard methods. Bone marrow transplantation. While under general anesthesia, 544 mL of marrow mixed with peripheral blood was aspirated from the posterior iliac crests of the normal syngeneic twin donor. A total of 3.6 × 10^9 mononuclear cells/kg of recipient were obtained. The aspirated marrow contained 142 mL of ABO compatible erythrocytes.
The patient had been treated unprocessed into the patient's central venous catheter. The donor stayed 1 day as an inpatient, the recipient none.

**Determination of twin monozygosity.** Evidence for monozygosity included physical appearance, identical HLA types, and identical alleles at six highly polymorphic loci typed by polymerase chain reaction (PCR).14

**Flow cytometry.** Analysis of expression of DAF (CD55) and MIRL (CD59) on erythrocytes, and CD16 and CD24 on PMN, was performed on whole blood using flow cytometry. Binding of purified monoclonal anti-CD55 IgG (Wako Bioproducts, Richmond, VA) and anti-CD59 IgG (Caltag, South San Francisco, CA) was detected indirectly with the R-phycocerythrin–labeled F(ab')2 fragment of donkey antinonuse IgG (Jackson ImmunoResearch, West Grove, PA). Directly labeled fluorescein isothiocyanate (FITC) conjugates of CD16 and CD24 were from Immunotech (Westbrook, ME). Light scatter gating of erythrocytes and PMN was used to collect more than 5,000 events on a four-decade log scale (Profile; Coulter, Hialeah, FL). Expression of DAF (CD55) and MIRL (CD59) on cultured T cells was analyzed by flow cytometry using published methods.15,16

**Cloning of PNH T cells.** The method of Endo et al17 was used. Cloning and sequencing of PIG-A cDNA using reverse-transcription PCR. Minor modifications of the methods reported by Takeda et al18 and Miyata19 were used.

**Direct sequencing of genomic DNA.** Six oligonucleotide primer sets (A-IT1 and B-1; A-1 and B-IT1; A-IT2 and B-IT2; A-IT3 and B-IT3; and A-IT4 and B-12) described by Iida et al20 were used to amplify regions 2 through 6 of PIG-A. These regions included 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 dye 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).

**Restriction analysis of PCR products.** The primer sets that flanked the appropriate regions of PIG-A cDNA (nucleotides 1095 to 1115 of the coding region and primer 12 described by Miyata et al19 or genomic DNA [A-IT3 and BIT-3]) were used to generate PCR products that included the mutation observed in the T-cell clone CD2E. The samples were incubated with BfaI (New England Biolabs, Beverly, MA) and analyzed by electrophoresis on agarose gels and ethidium bromide staining.

**Analysis of the PIG-A mutation in PNH T-cell clones CD2A, CD2D, CD2F, CD3G, and CD3H.** A primer set consisting of nucleotides 61 to 81 (sense primer) and 179 to 199 (antisense primer) was used to amplify a 141-bp PCR product containing the mutation. Aliquots of [α-32P]dATP (3,000 Ci/mmol/L) (NEN Research Products, Boston, MA) were included in the PCR reaction mixture, and the PCR products were subsequently analyzed using 6% denaturing polyacrylamide gel electrophoresis and autoradiography.

**Buccal cells.** DNA was isolated from buccal cells according to the protocol provided by the manufacturer using the Puregene kit (Gentra Systems, Minneapolis, MN).

**Analysis of X-chromosome inactivation.** The method of Allen et al19 was used.

**RESULTS**

**Case report.** The patient is a girl (date of birth, 10/19/83) who was well until age 9 years 3 months (January 1993) when, in association with an upper respiratory tract infection and stomatitis, she noted severe substernal chest pain and black urine. Because of recurrent urinary tract infection, the patient had been treated for approximately 6 years with 25 mg/d of nitrofurantoin (Macrodantin; Procter & Gamble Pharmaceuticals, Norwich, NY). The drug was discontinued. Over the next 10 months, she experienced three additional episodes of severe substernal chest pain followed by hemoglobinuria and lethargy lasting several days. The symptoms appeared to be precipitated by minor infections. In November 1993, following an episode of hemoglobinuria, the patient was found to have a positive Ham's test and a positive sucrose lysis test. At that time, both the white blood cell count and platelet count were normal, the hemoglobin concentration was 11.9 g/dL, and the serum lactate dehydrogenase (LDH) level was 1,734 U/L (normal range, 135 to 310 U/L). The diagnosis of PNH was confirmed by flow cytometry that showed abnormal expression of GPI-AP on the peripheral blood cells. The patient continued to experience the same constellation of symptoms intermittently over the next several months. HLA-typing and DNA analysis showed that her twin sister was genetically identical, and flow cytometry showed normal expression of GPI-AP on the peripheral blood cells of the twin. On April 5, 1994, the patient was infused with bone marrow cells that had been harvested from her twin that same day.

**Clinical response to syngeneic marrow infusion.** Eight days before transplant, the hemoglobin concentration was 10.9 g/dL and the serum LDH level was 1,760 U/L. Four months posttransplant, the hemoglobin concentration was 14.6 g/dL and the serum LDH level was 419 U/L. For the next 13 months, the patient remained asymptomatic, despite having had infections that would have previously precipitated clinical symptoms of PNH. The hemoglobin concentration remained stable, but a gradual increase in the serum LDH level was observed (448 U/L 8 months posttransplant; 498 U/L 14 months posttransplant). Approximately 16.5 months posttransplant, the patient noted recurrent episodes of nocturnal substernal chest pain. Concordant with the onset of symptoms, both an increase in the serum LDH level to 645 U/L and a decrease in the plasma hemoglobin concentration to 13.7 g/dL were observed.

**Effects of marrow infusion on GPI-AP expression.** Before transplant, approximately 50% of the patient's erythrocytes showed normal CD55 (DAF) expression, while approximately 25% showed normal CD59 (MIRL) expression (Fig 1). Four months posttransplant, normal CD55 expression increased to 80%, and normal CD59 expression increased to 65%. Through 13 months of additional observation, a decline in the percentage of erythrocytes with normal CD55 expression was observed, but CD59 expression during this period appeared stable. Seventeen months posttransplant, normal CD55 and CD59 expression was observed on 60% and 68% of the erythrocytes, respectively.

Analysis by flow cytometry of the patient’s peripheral blood PMN also showed an initial increase in GPI-AP expression following infusion of the syngeneic marrow (Fig 2). One day before transplant, 9% and 14% of the PMN expressed normal amounts of CD16 (FcyRIIIb) and CD24, respectively. Four months posttransplant, normal expression of CD16 was observed on 40% of the PMN, while normal expression of CD24 was observed on 57%. Thereafter, a gradual decline in expression of the two GPI-AP was noted.
Fig 1. Flow cytometry of erythrocyte GPI-AP expression before and after marrow infusion. Peripheral blood cells were obtained from the patient just prior to the marrow infusion and at various times afterwards (indicated on left). Expression of GPI-AP was determined by incubating erythrocytes with either monoclonal anti-DAF (CD55) or monoclonal anti-MIRL (CD59). Next, the cells were incubated with phycoerythrin (PE)-conjugated antibody against mouse IgG and subsequently analyzed by flow cytometry.

Fig 2. Flow cytometry of PMN before and after the marrow infusion. PMN were isolated from the peripheral blood and expression of GPI-AP was analyzed by flow cytometry using labeled anti-CD16 (FcyR1lb) and anti-CD24.

Development of PNH T-cell clones. To determine the PIG-A mutations that produced the GPI-AP deficiency in this patient, T-lymphocyte clones derived from the patient’s peripheral blood pretransplant were established. Flow cytometry (Fig 3) showed that some of the clones had normal expression of GPI-AP (eg, clones CD1D). However, other clones showed abnormal GPI-AP expression (eg, CD2A, CD2D, and CD2E). Among the T-cell clones with abnormal GPI-AP expression, the phenotype was similar. When the peripheral blood lymphocytes of the patient’s twin sister were subjected to the same type of analysis, no GPI-AP-deficient lymphocyte clones were identified (not shown).

Analysis of PIG-A. By using reverse-transcription (RT)-PCR, PIG-A cDNA from the T-cell clones was amplified and the nucleotide sequence subsequently determined. No mutations were found in the PIG-A cDNA derived from the T-cell clones with normal GPI-AP expression (represented by CD1D, Fig 3, and CM1C, not shown). These results imply that clones with normal GPI-AP expression are the progeny of stem cells with normal PIG-A.

In contrast, PIG-A mutations were identified in all clones with abnormal GPI-AP expression. Clones CD2A and CD2D (Fig 3), along with clones CD2F, CD3G, and CD3H (not shown), were found to have a 2-bp (AT) insertion between nucleotides 98 and 106 that caused a frameshift and introduced a premature stop signal at codon 61. This mutation provides a plausible explanation for the observed GPI-AP deficiency, since 423 (of 484) amino acids would be deleted from the protein product of this abnormal gene. Direct sequencing of genomic DNA from these clones demonstrated that the insertion mutation was heterozygous (not shown). This conclusion was supported by PCR analysis of genomic DNA (Fig 4). When the PIG-A from the clones known to contain the mutant allele (represented by CD2A, CD2D, and
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a) Anti-DAF (CD55) Anti-MIRL (CD59)

![Flow cytometry of T-cell clones.](image)

Fig 3. Flow cytometry of T-cell clones. T lymphocytes from the patient were cloned by limiting dilution, and expression of GPI-AP was analyzed by flow cytometry using anti-DAF (CD55) and anti-MIRL (CD59) as primary antibodies.

CD2F) was analyzed, two bands were observed (the lower band representing the wild-type allele and the upper band representing the allele with the 2-bp insertion). Only the wild-type allele was observed in clones CD1D, CM1C, and CD2E, demonstrating that the mutation was not inherited (Fig 4). No evidence of the mutation was observed in the genomic DNA derived from peripheral blood cells obtained either before or after the transplantation (Fig 4). Inasmuch as the pretransplant DNA that was analyzed in these experiments was derived from whole blood containing lymphocytes, these result suggest that the proportion of peripheral blood cells bearing the 2-bp insertion is below the threshold of detection. This finding implies that the abnormal stem cell from which these mutant cells arose was producing a relatively small number of progeny.

Clone CD2E showed aberrant expression of GPI-AP (Fig 3), but the PIG-A mutation was different from that of the other abnormal clones (Fig 4). Sequence analysis of PIG-A cDNA showed a G-to-A transition at position 1160. This substitution changed codon 387 from the wild-type TGG (Trp) to the mutant TAG (nucleotides involved in the transition shown in bold), thereby introducing a premature stop signal. This nonsense mutation could account for the abnormal GPI-AP expression by CD2E, since it results in truncation of 97 residues from the COOH-terminal portion of the PIG-A protein.

The G-to-A transition introduced a new BfaI restriction site. To characterize further the mutation in clone CD2E, PCR primers that flank the site of the substitution were used to amplify a 418-bp product from PIG-A cDNA. After incubation with BfaI, the PCR product from from CD2E cDNA was completely digested (Fig 5A), confirming that the mutant allele was located on the active X chromosome. In contrast, the new restriction site was not contained in the PIG-A cDNA from clones CD1D and CD2F (Fig 5A), showing that the active allele from those clones did not contain the CD2E mutation. Analysis of PIG-A cDNA derived from PMN isolated from the patient’s peripheral blood 14 months posttransplant showed that a portion contained the BfaI restriction site. These results indicate that the mutant stem cell was still active more than 1 year after infusion of the syngeneic marrow. The restriction pattern of PIG-A cDNA from PMN isolated from the patient’s twin sister was normal (Fig 5A), demonstrating that the mutant stem cell was not of donor origin.

To confirm that the mutation was present in genomic DNA, PCR primers that flanked the mutation were used to amplify a 581-bp segment of PIG-A. After incubation with BfaI, the PCR products from the normal control DNA and from DNA from clones CD1D and CD2A were undigested, whereas a portion of the CD2E product was cleaved into fragments of 494 and 87 bp (Fig 5B). Together, these results indicated the following: (1) the mutation in CD2E is heterozygous; and (2) the mutation is somatic in origin rather than inherited.

![Demonstration of a 2-bp insertion mutation of PIG-A.](image)

Fig 4. Demonstration of a 2-bp insertion mutation of PIG-A. PCR primers that flanked the region of PIG-A containing the 2-bp insertion that had been identified in some of the T-cell clones with abnormal GPI-AP expression were used to amplify DNA. Subsequently, the PCR products were analyzed by using denaturing polyacrylamide gel electrophoresis and autoradiography. The source of the DNA is indicated above the appropriate lane. Dnr indicates that the DNA was obtained from the peripheral blood cells of the patient’s twin sister (the donor), PNH indicates that the DNA was obtained from the patient’s peripheral blood cells. For both pretransplant samples, the DNA was isolated from whole blood. The posttransplant DNA was derived from isolated PMN.
Fig 5. Bfai restriction analysis. (A) Analysis of RT-PCR products. cDNA was prepared from PNH T-cell clones CD1D, CD2E, and CD2F and from PMN isolated from the peripheral blood of the patient (PNH) and her twin sister (Dnr) 14 months posttransplant. A primer set that flanks the site of the mutation in CD2E was used to amplify a 418-bp segment of PIG-A cDNA. After incubation with Bfai, the samples were analyzed by agarose gel electrophoresis and ethidium bromide staining. Bfai cleaves the wild-type product into fragments of 406 and 12 bp. The 12-bp fragment is not visible on the gel. The mutation in clone CD2E introduces a restriction site that produces cleavage fragments of 306 and 100 bp. Left, the position of the size standard (St) is indicated. Right, the sizes of the cleavage fragments are indicated. (B) Analysis of genomic PCR products. DNA was isolated from T-cell clones CD1D, CD2A, and CD2E, and from normal (NL) PMN. A primer set that flanks the mutation in CD2E was used to amplify a 581-bp segment of PIG-A. The PCR products were incubated with Bfai and subsequently analyzed by agarose gel electrophoresis and ethidium bromide staining. The wild-type PCR product does not contain a Bfai restriction site. The mutation creates a Bfai restriction site such that the PCR product is cleaved into fragments of 494 and 87 bp. Left, the position of the size standard (St) is indicated. Right, the sizes of the cleavage fragments are indicated.

Analysis of the effects of syngeneic marrow infusion on hematopoiesis. Flow cytometry showed that the proportion of peripheral blood cells with normal GPI-AP expression increased following the transplant (Figs 1 and 2), implying engraftment of donor cells. To investigate the effects of transplantation on the contribution of the abnormal stem cells to hematopoiesis, we took advantage of the Bfai restriction site introduced by the CD2E mutation. DNA was isolated from the peripheral blood of the patient and her twin sister (the donor) 2 months pretransplant and 14 months posttransplant and used for PCR amplification of the region of PIG-A containing the mutation. DNA from clone CD2E served as the control. Following incubation with Bfai, the donor PCR product was undigested while the restriction pattern of the PCR product from CD2E was consistent with a heterozygous mutation (Fig 6). In the case of DNA from the patient, the restriction pattern posttransplant was similar to that of the pattern pretransplant, indicating that the relative contribution of the mutant stem cell to hematopoiesis was minimally affected by the transplantation.

To investigate further the effects of the transplant on hematopoiesis, the pattern of X-chromosome inactivation was analyzed. In these experiments, genomic DNA was incubated with buffer or with buffer containing the methylation-sensitive restriction enzyme HpaII. Next, a highly polymorphic region of the human androgen receptor gene (HUMARA) located on the X chromosome was amplified by PCR. The PCR primers flank two HpaII sites near the polymorphic region of HUMARA. Thus, only the inactivated (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. For the results to be informative, the alleles must be sufficiently polymorphic to allow resolution when analyzed by denaturing polyacrylamide gel electrophoresis.

In the buffer control samples [the (−) samples] from the patient and the donor, both the active and the inactive genes are amplified (Fig 7). In the HpaII-treated samples [the (+) samples] from the donor, both alleles are represented equally. This pattern of random X inactivation is indicative of polyclonal hematopoiesis. In contrast, analysis of DNA from the peripheral blood cells of the patient before transplantation showed a nonrandom pattern of X inactivation (Fig 7). Analysis of the patient’s peripheral blood cells after transplant showed a pattern of nonrandom X inactivation that was similar to that observed pretransplant. Since the donor cells displayed a pattern of random X inactivation, this observation implies...
that the number of cells that engrafted was not sufficient to shift the X-inactivation pattern in the hematopoietic cells of the patient from nonrandom to random. These results support the hypothesis that the clinical benefit experienced by the patient following the transplant resulted from engraftment of a relatively small number of donor cells.

Analysis of the pattern of X inactivation in hematopoietic and buccal cells. The observation that the pattern of X inactivation in the peripheral blood cells of the patient was nonrandom could be explained either by skewing of X inactivation or by dominance of hematopoiesis by a stem cell in which the allele represented by the upper band is inactive (Fig 7). To distinguish between these two possibilities, analysis of X-chromosomal inactivation in other somatic tissue was compared with that of hematopoietic cells. The pattern of X inactivation of buccal mucosal cells from the patient was identical to that of her PMN (Fig 8A). Thus, the nonrandom pattern of X inactivation observed in the peripheral blood cells is not due to clonal dominance of hematopoiesis, but rather to marked skewing of X inactivation. These findings also support the hypothesis that the pattern of X inactivation is stochastic rather than genetically determined, since the patient’s twin sister has a random pattern (Fig 7). Although a different allele is inactive, the patient’s mother also has a pattern of nonrandom X inactivation (Fig 8A), that, like the patient, is due to extreme skewing of X inactivation, rather than to monoclonality of hematopoiesis. These results underscore the importance of analyzing the pattern of X inactivation in additional somatic tissues before attributing a pattern of nonrandom X inactivation observed in hematopoietic tissues to clonal dominance.

To insure that the buccal cells were not contaminated with hematopoietic tissues, DNA was analyzed for PIG-A mutations by restriction analysis. The region of PIG-A containing the CD2E mutation (the G-to-A substitution at nucleotide position 1160) was amplified, and the PCR products were incubated with BfaI, demonstrating the presence of mutant cells. In contrast, the PCR product derived from the DNA of the patient’s buccal cells was not cleaved by BfaI. These findings show that the DNA isolated...
Fig 8. Analysis of DNA from buccal mucosal cells and PMN. (A) Pattern of X inactivation. PMN-1 were isolated 14 months posttransplant and PMN-2 were isolated 17 months posttransplant as were the buccal cells. The source of the cells (Mother or Patient) is indicated. The DNA was isolated from the samples and incubated with buffer (−) or with buffer containing HpaII (+). The treated samples were used as template for PCR amplification of a highly polymorphic region of the androgen receptor gene (HUMARA) that is located on the X chromosome. The PCR products were subsequently analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. (B) BfaI restriction analysis of genomic PCR products. PMN and buccal cells were obtained 17 months posttransplant. The source of the cells (Patient or Mother) is indicated. DNA was isolated from the samples and used as template for PCR. A primer set that flanks the CDSE mutation (G-to-A transition at positions 1160) was used to amplify a 581-bp segment of PIG-A. The PCR products were incubated with buffer (−) or with buffer containing BfaI (+) and analyzed by agarose gel electrophoresis and ethidium bromide staining. The CDSE mutation creates a BfaI restriction site such that the PCR product is cleaved into fragments of 494 and 87 bp. The 87-bp fragment is not shown. Left, the position of the size standard (St) is indicated. Right, the size (in bp) of the PCR products is indicated.

from the buccal cells was not contaminated with hematopoietic cell DNA and confirm that PNH is due to somatic mutations involving the hematopoietic stem cell rather than to germ-line mosaicism.

DISCUSSION

Here we report the effects of infusion of syngeneic bone marrow on a patient with PNH. Following the transplant, the patient’s symptoms resolved, and laboratory evidence of amelioration of intravascular hemolysis was observed in that the serum LDH level decreased from 1,760 U/L 8 days pretransplant to 419 U/L 4 months posttransplant. Our studies indicated that donor stem cells engrafted and proliferated initially. This conclusion is based on the observation that following the infusion, the proportion of erythrocytes and granulocytes with normal GPI-AP expression increased (Fig 1 and 2). However, subsequently, symptoms recurred concordant with evidence of worsening hemolysis and an increase in the proportion of GPI-AP-negative peripheral blood cells (Fig 2). These results are consistent with the hypothesis that the mutant stem cells have a relative survival advantage.

That the patient, but not her sister, was placed on chronic bacterial suppressive therapy because of recurrent urinary tract infections suggests a plausible explanation for why only one of this pair of monozygotic twins (who had never lived apart) developed PNH. Nitrofurantoin is associated with a variety of hematological abnormalities, including rare reports of aplastic anemia and megaloblastic anemia, and PNH often develops in association with other diseases that affect the hematopoietic stem cell. Thus, it is possible that the nitrofurantoin injured the stem cells, thereby creating an environment in which PNH could evolve. While the mechanism by which PNH develops in the setting of marrow injury is speculative, a plausible hypothesis is that under some conditions, GPI-AP-deficient cells are selected because they have a survival advantage. For example, nitrofurantoin could initiate either a cellular or a humoral immune-mediated process in which a GPI-AP is the target antigen. Under those circumstances, GPI-AP-deficient stem cells would escape injury and consequently dominate hematopoiesis.

Our results favor the hypothesis that GPI-AP-deficient stem cells have a survival advantage relative to stems cells with normal GPI-AP expression because the proportion of hematopoietic cells with normal GPI-AP expression declined gradually after the initial period of engraftment and proliferation of donor cells (Figs 1 and 2). The mechanism that effected this survival advantage is obscure; however, it appeared to persist independent of nitrofurantoin, since the drug was discontinued approximately 14 months before transplant. Further, the pathological process must be insidi-
ous in that evidence of disease progression accumulated slowly during the 17 months of observation posttransplant (Figs 1 and 2).

Here, we have identified two discrete PIG-A mutations occurring in the same patient. Both mutations affect GPI-AP expression in an equivalent fashion (Fig 3). However, one of the mutations (the G-to-A substitution that introduces a BfAI restriction site) appears to affect a stem cell that contributes significantly to hematopoiesis (Fig 5), while the stem cell affected by the other mutation (the 2-bp insertion) appears relatively quiescent (Fig 4). Together, these results indicate that the PIG-A mutation alone does not determine the proliferative capacity of the stem cell. If the PIG-A mutation per se endowed mutant stem cells with a proliferative advantage, evidence of clonal dominance of hematopoiesis by cells with the 2-bp insertion, as well as by cells with the G-to-A substitution, should be observed. Thus, the hypothesis that PNH arises because GPI-AP-deficient cells have a growth advantage is not supported by these studies. Rather, it appears that proliferative capacity is an intrinsic property of the stem cell and that GPI-AP deficiency bestows a conditional survival advantage, rather than a conditional proliferative advantage on the affected stem cell.

The observation that individual patients with PNH can have more than one PIG-A mutation has been reported. In one study, we identified four distinct somatic mutations of PIG-A in the hematopoietic cells of a single patient. The interpretation of these findings is open to speculation. Whether the mutational rate is abnormally high in conditions contributing significantly to hematopoiesis (Fig 3), while the stem cell affected by the other mutation (the 2-bp insertion) appears relatively quiescent (Fig 4). Together, these results indicate that the PIG-A mutation alone does not determine the proliferative capacity of the stem cell. If the PIG-A mutation per se endowed mutant stem cells with a proliferative advantage, evidence of clonal dominance of hematopoiesis by cells with the 2-bp insertion, as well as by cells with the G-to-A substitution, should be observed. Thus, the hypothesis that PNH arises because GPI-AP-deficient cells have a growth advantage is not supported by these studies. Rather, it appears that proliferative capacity is an intrinsic property of the stem cell and that GPI-AP deficiency bestows a conditional survival advantage, rather than a conditional proliferative advantage on the affected stem cell.

The observation that individual patients with PNH can have more than one PIG-A mutation has been reported. In one study, we identified four distinct somatic mutations of PIG-A in the hematopoietic cells of a single patient. The interpretation of these findings is open to speculation. Whether the mutational rate is abnormally high in conditions predisposing to PNH or whether GPI-AP-deficient stem cells are present in the marrow before the onset of the disease process are debatable issues. Further, the two hypotheses are not mutually exclusive. However, that multiple PIG-A mutations are observed in the same patient is consistent with the interpretation that some aspect of GPI-AP deficiency is advantageous and provides the selective pressure that results in the emergence of abnormal stem cells that ultimately dominate hematopoiesis.

Two lines of evidence indicate that the number of stem cells that engrafted was relatively small. First, BfAI restriction analysis of PIG-A from peripheral blood cells before and after the transplant showed a similar pattern (Fig 6). Second, the nonrandom pattern of X inactivation in the cells of the patient was not shifted toward a random pattern following the transplant (Fig 7). The reason for the apparently low level of engraftment is speculative. Conceivably, the patient’s marrow may have been too cellular to provide adequate sites for the transplanted marrow to engraft optimally. Alternatively, the number of engrafted cells may be similar to that which occurs following allogeneic marrow transplantation, since clonal hematopoiesis of donor origin has been reported in that situation. This observation implies that a single or a few pluripotent stem cells are responsible for maintaining hematopoiesis after transplantation. That the relatively small number of stem cells that engrafted had such a significant effect on the proportion of peripheral blood cells with normal GPI-AP expression suggests that the residual number of functionally normal hematopoietic stem cells of recipient origin must have been small. Thus, in addition to mutations of PIG-A, a deficiency of normal stem cells appears to be essential for the clinical manifestation of PNH to become apparent.

Earlier studies by others have concluded that PNH is a monoclonal disease. However, recent experiments from this laboratory have shown that PNH cells are polyclonal in some cases. In the present study, the pattern of X inactivation observed in the peripheral blood cells of our patient was consistent with clonal dominance of hematopoiesis (Fig 7); however, analysis of buccal mucosal cells demonstrated that extreme skewing accounted for the nonrandom pattern of X inactivation (Fig 8A). This finding emphasizes the importance of analyzing other somatic tissues before reaching conclusions about clonality of hematopoietic cells based on the pattern of X inactivation. Further, in cases of extreme skewing of X inactivation, there is a high probability that the pattern of X inactivation will be identical among individual affected cells, even when the PIG-A mutations occur independently. In such cases, a uniform pattern of X inactivation is not unequivocal evidence of monoclonality.

This is the fifth reported case of syngeneic bone marrow transplantation without pretransplant conditioning in a patient with PNH. Of the five patients so treated, four (including the present patient) had evidence of a return to abnormal hematopoiesis following transplant. Together, these studies provide in vivo evidence in support of the hypothesis that GPI-AP-deficient stem cells dominate hematopoiesis in PNH, because they have a survival advantage relative to normal stem cells. Future studies will be aimed at characterizing the pathological process that exerts the selective pressure and at identifying the GPI-AP that is the target of that process.

ACKNOWLEDGMENT

The authors thank Margaret Robertson of the University of Utah Health Sciences Center Sequencing Facility for analysis of genomic DNA. Oligonucleotides were prepared by the DNA/Peptide Core Facility of the University of Utah supported in part by Grant No. CA42014 from the National Cancer Institute. Analysis by flow cytometry of the T cell clones was performed at the Huntsman Cancer Institute Flow Cytometry Core Facility of the University of Utah. The authors thank Dr Roberta Adams (University of Utah School of Medicine) for referring the patient and Dr Elizabeth M. Prystas (LDS Hospital, Salt Lake City, UT) for critically reviewing the manuscript. The authors are indebted to the patient, her sister, and her mother. Without their cooperation, these studies would not have been possible.

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Syngeneic bone marrow transplantation without conditioning in a patient with paroxysmal nocturnal hemoglobinuria: in vivo evidence that the mutant stem cells have a survival advantage

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