Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by the presence in the patient’s hematopoietic system of a large cell population with a mutation in the X-linked PIG-A gene. Although this abnormal cell population is often found to be monoclonal, it is not unusual that 2 or even several PIG-A mutant clones coexist in the same patient. Therefore, it has been suggested that the PIG-A gene may be hypermutable in PNH. By a method we have recently developed for measuring the intrinsic rate of somatic mutations (μ) in humans, in which PIG-A itself is used as a sentinel gene, we have found that in 5 patients with PNH, μ ranged from 1.24 × 10^{-7} to 1.12 × 10^{-7}, against a normal range of 2.4 × 10^{-7} to 29.6 × 10^{-7} mutations per cell division. We conclude that genetic instability of the PIG-A gene is not a factor in the pathogenesis of PNH.

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Study design

Patients with “classic PNH” (Table 1) referred to Memorial Sloan-Kettering Cancer Center were recruited to the study and provided blood samples with informed consent on a protocol approved by the Institutional Review Board. Immortalized B-lymphoblastoid cell lines (BLCLs) were generated as previously described. Typically, BLCL populations from PNH patients either are entirely GPI− or have both GPI+ and GPI− subpopulations. We were interested in the GPI− cells from PNH patients, because they would provide a phenotypic marker for spontaneous PIG-A mutations: a change from the GPI− to a GPI+ phenotype. From 5 PNH patients we could generate GPI− BLCLs (Table 2). The mutation rate in the PIG-A gene was determined as recently described. This method takes advantage of flow sorting to eliminate preexisting mutants from the BLCL population, expansion of the collected GPI− cells in vitro, and analysis of a large number of cells to identify rare GPI− cells that have arisen due to spontaneous mutations in PIG-A in vitro.

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Results and discussion

We used a combination of several antibodies that bind to distinct GPI-linked proteins. GPI− cells in a mixture with GPI+ cells are thus readily identified (Figure 1A). Occult GPI− cells arising spontaneously within a BLCL from a healthy donor are also clearly distinguished from the much larger GPI+ population from which they arise (Figure 1B). Using a cell line from a patient with ataxia-telangiectasia as a positive control for hypermutability, we observed an increased frequency of spontaneously arising GPI− cells (Figure 1C).

We used this combination of anti-CD48, anti-CD55, and anti-CD59, PE-conjugated rabbit anti–mouse immunoglobulin antibody, and anti-HLA-DR–FITC. The number of events is shown in each quadrant. The presence of an expanded GPI− population and the finding of several different simultaneous or successive clones with distinct PIG-A mutations had been regarded as indicative of an increased mutation rate. The increased frequency of circulating HPRT mutants and glycophorin A variants in PNH had also been taken as evidence of hypermutability.10,20,22,23 Chen et al21 replicated such findings but provided data implicating increased cell divisions rather than hypermutability as the explanation. Our work now addresses the issue directly in 2 ways: (1) We use the same gene that is mutated in PNH as our sentinel gene, and (2) we eliminate preexisting mutants from the population to control for cell divisions and to calculate the mutation rate. From our findings, we conclude that an increased rate of mutation in PIG-A is not a part of the pathogenesis of PNH.

Our finding of a normal mutation rate in PNH is consistent with the observation that patients with this disorder are not particularly prone to cancer,11 whereas patients with inherited genomic instability (eg, ataxia-telangiectasia and Fanconi anemia) are not prone to PNH. Thus, hypermutability of the PIG-A gene is neither necessary nor sufficient for the development of PNH. While an increased number of cell divisions could result in an increased frequency of mutants,28 this must not be sufficient to result in PNH, because

Table 1. Patients recruited to the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y/sex</th>
<th>History of aplastic anemiaa</th>
<th>Thrombosis</th>
<th>Hemoglobin level, g/dL</th>
<th>Reticulocyte count, %</th>
<th>WBC count, × 10⁹/L</th>
<th>Platelet count, × 10¹²/L</th>
<th>% PNH red blood cells†</th>
<th>% PNH II red blood cells†</th>
<th>% PNH polymorphonuclear leukocytes†</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29/F</td>
<td>Yes</td>
<td>No</td>
<td>5.4</td>
<td>2.6</td>
<td>2.5</td>
<td>19</td>
<td>20</td>
<td>5</td>
<td>70</td>
<td>ATG</td>
</tr>
<tr>
<td>2</td>
<td>51/M</td>
<td>Yes</td>
<td>No</td>
<td>9.7</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>9</td>
<td>24</td>
<td>74</td>
<td>ATG/CSA</td>
</tr>
<tr>
<td>3</td>
<td>40/F</td>
<td>No</td>
<td>Yes</td>
<td>12.4</td>
<td>2.2</td>
<td>4.2</td>
<td>74</td>
<td>6</td>
<td>6</td>
<td>52</td>
<td>Enoxaparin</td>
</tr>
<tr>
<td>4</td>
<td>74/M</td>
<td>No</td>
<td>No</td>
<td>9.9</td>
<td>8.1</td>
<td>3.4</td>
<td>92</td>
<td>34</td>
<td>9</td>
<td>84</td>
<td>Transfusion</td>
</tr>
<tr>
<td>5</td>
<td>33/F</td>
<td>No</td>
<td>No</td>
<td>9.4</td>
<td>20.2</td>
<td>3.7</td>
<td>263</td>
<td>28</td>
<td>24</td>
<td>83</td>
<td>Transfusion</td>
</tr>
</tbody>
</table>

To convert hemoglobin level from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

aPrior or concurrent history of clinically recognized aplastic anemia.

bFraction of PNH red cells and granulocytes was performed as previously described.25

*Prior or concurrent history of clinically recognized aplastic anemia.

†Red cells with a partial deficiency in CD59 expression.

Table 2. Analysis of cell lines from patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>d*</th>
<th>No. of GPI− cells</th>
<th>No. of GPI+ cells</th>
<th>f × 10⁹</th>
<th>μ × 10⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.24</td>
<td>4</td>
<td>846 083</td>
<td>4.73</td>
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<td>2</td>
<td>10.6</td>
<td>8</td>
<td>792 311</td>
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<tr>
<td>3</td>
<td>9.5</td>
<td>1</td>
<td>849 779</td>
<td>1.18</td>
<td>1.24</td>
</tr>
<tr>
<td>4</td>
<td>6.49</td>
<td>2</td>
<td>934 988</td>
<td>1.36</td>
<td>2.10</td>
</tr>
<tr>
<td>5</td>
<td>6.07</td>
<td>5</td>
<td>2 342 270</td>
<td>2.13</td>
<td>3.51</td>
</tr>
</tbody>
</table>

*Number of cell divisions occurring in culture after sorting. In cell lines from the 5 patients, we collected by flow sorting a mean of 2 × 10³ live cells, which proliferated in culture for a mean of 32 days, undergoing a mean of 7.4 cell divisions.

†Frequency (per 10⁹) of GPI− cells after expansion after sorting: f = number of GPI− cells / number of GPI+ cells.

‡Mutation rate (mutations per 10⁷ cell divisions): μ = f − d.

Figure 1. Flow cytometric dot plot analysis for the calculation of f and μ. BLCLs were analyzed after staining sequentially with a mixture of unconjugated anti–human CD48, CD55, and CD59, PE-conjugated rabbit anti–mouse immunoglobulin antibody, and anti-HLA-DR–FITC. The number of events is shown in each quadrant. The mutant frequency (f) is calculated as the number of GPI− cells divided by the number of GPI+ cells. (A) Normal GPI− cells mixed with GPI+ cells from a patient with PNH. (B) BLCLs from healthy donor 1, where the frequency of mutants is 22 × 10⁻⁷ (C) BLCLs from a patient with ataxia-telangiectasia, with increased GPI− cells (f = 245 × 10⁻⁵). (D-F) To calculate the mutation rate, preexisting mutants are eliminated from the population by collecting the upper 50th percentile of the distribution curve after staining with anti-CD59. The collected GPI− cells are then returned to culture and expanded. The mutation rate is determined by the formula μ = f − d, where d represents the number of cell divisions occurring in vitro after sorting.24 (D) Analysis of a BLCL from healthy donor 2 after expansion after flow sorting. f = 4.73 × 10⁻⁶, d = 4.24 cell divisions, and μ = 11.2 × 10⁻⁷ mutations per cell division. (E-F) Analyses of 2 representative cell lines from PNH patients. (E) Analysis of a BLCL from PNH patient 1 after expansion after flow sorting. f = 5.05 × 10⁻⁶, d = 10.6 cell divisions, and μ = 4.76 × 10⁻⁷ mutations per cell division.
PNH is not seen after recovery from chemotherapy-induced marrow injury. Therefore, we conclude that the expansion of PIG-A mutant clones occurs as a result of selection in favor of the mutants, which occurs only in certain forms of marrow injury, such as in aplastic anemia.\(^7\)\(^9\) Having ruled out hypermutability in PNH, we predict that the direct measurement of the mutation rate will be useful in elucidating the pathophysiology of other clonal hematologic disorders.

References

The mutation rate in PIG-A is normal in patients with paroxysmal nocturnal hemoglobinuria (PNH)

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