Impaired Hematopoiesis in Paroxysmal Nocturnal Hemoglobinuria/Aplastic Anemia Is Not Associated With a Selective Proliferative Defect in the Glycosylphosphatidylinositol-Anchored Protein-Deficient Clone

By Jaroslaw P. Maciejewski, Elaine M. Sloand, Tadatsugu Sato, Stacie Anderson, and Neal S. Young

Paroxysmal nocturnal hemoglobinuria (PNH) results from somatic mutations in the PIG-A gene, leading to poor presentation of glycosylphosphatidylinositol (GPI)-anchored surface proteins. PNH frequently occurs in association with suppressed hematopoiesis, including frank aplastic anemia (AA). The relationship between GPI-anchored protein expression and bone marrow (BM) failure is unknown. To assess the hematopoietic defect in PNH, the numbers of CD34+ cells, committed progenitors (primary colony-forming cells [CFCs]), and long-term culture-initiating cells (LTC-ICs; a stem cell surrogate) were measured in BM and peripheral blood (PB) of patients with PNH/AA syndrome or patients with predominantly hemolytic PNH. LTC-IC numbers were extrapolated from secondary CFC numbers after 5 weeks of culture, and clonogenicity of LTC-ICs was determined by limiting dilution assays. When compared with normal volunteers (n = 13), PNH patients (n = 14) showed a 4.7-fold decrease in CD34+ cells and an 8.2-fold decrease in CFCs. LTC-ICs in BM and in PB were decreased 7.3-fold and 50-fold, respectively. Purified CD34+ cells from PNH patients had markedly lower clonogenicity in both primary colony cultures and in the LTC-IC assays. As expected, GPI-anchored proteins were decreased on PB cells of PNH patients. On average, 23% of monocytes were deficient in CD14, and 47% of granulocytes and 58% of platelets lacked CD16 and CD55, respectively. In PNH BM, 27% of CD34+ cells showed abnormal GPI-anchored protein expression when assessed by CD59 expression. To directly measure the colony-forming ability of GPI-anchored protein-deficient CD34+ cells, we separated CD34+ cells from PNH patients for the GPI- and GPI-phenotype; CD59 expression was chosen as a marker of the PNH phenotype based on high and homogeneous expression on fluorescent staining. CD34+CD59- and CD34+CD59+ cells from PNH/AA patients showed similarly impaired primary and secondary clonogenic efficiency. The progeny derived from CD34+CD59- cells were both CD59- and CD55-.

A very small population of CD34+CD59- cells was also detected in some normal volunteers; after sorting, these CD34+CD59- cells formed normal numbers of colonies, but their progeny showed lower CD59 levels. Our results are consistent with the existence of PIG-A-deficient clones in some normal individuals. In PNH/AA, progenitor and stem cells are decreased in number and function, but the proliferation in vitro is affected similarly in GPI-protein-deficient clones and in phenotypically normal cells. As measured in the in vitro assays, expansion of PIG-A-deficient clones does not appear to be caused by an intrinsic growth advantage of cells with the PNH phenotype.

© 1997 by The American Society of Hematology.

From the Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD.

Submitted May 30, 1996; accepted September 26, 1996.

Address reprint requests to Neal S. Young, MD, 10 Center Dr MSC 1652, Building 10, Room 7C103, Bethesda, MD 20892-1652.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.

0006-4971/97/8904-0001$3.00/0

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
and Agranulocytosis 20; severity was classified by the criteria of Cam.

CD34 MoAb. In some experiments Cy-PE ± labeled CD34 MoAb and PB count criteria of the International Study of Aplastic Anemia with fluorescein isothiocyanate (FITC)-conjugated HPCA-1 anti-
nnosis of AA was established according to the BM biopsy specimen man CD59 (Research Diagnostics Inc, Flanders, NJ) was combined and had normocellular or hypercellular BM morphology. The diag-

nosis of AA, lacked significant neutropenia or thrombocytopenia, milliliter of PB. For the measurement of the expression of GPI-

The PNH group consisted of patients with predominantly hemolytic PB. The frequency of CD34 was established by clinical ®ndings and results of the Ham test BMMNCs. BM cells were incubated with appropriate directly conju-

gated molecules, a panel of MoAbs was used. PE-labeled antihu-

history of AA, lacked signi®cant neutropenia or thrombocytopenia, milliliter of PB. For the measurement of the expression of GPI-

was established by clinical ®ndings and results of the Ham test BMMNCs. BM cells were incubated with appropriate directly conju-

gated molecules, a panel of MoAbs was used. PE-labeled antihu-

All the patients received transfusion in the course of the disease; no transfusions were administered in the period before immediate PB examination.

Abbreviations: ANC, absolute neutrophil count; Plt, platelets; AML, acute myelogenous leukemia; Hemol, hemolysis; Normo, normocellular BM; Hypo, hypocellular BM; Hyper, hypercellular BM.

* Time from the initial diagnosis.

tors, we directly compared the clonogenicity of GPI-de®cient CD34+ cells (PNH phenotype; CD59-) with that of normal CD34+ cells (CD59+) obtained from individual patients with PNH using primary colony assays and long-term BM culture (LTBMC).

MATERIALS AND METHODS

BM samples were obtained from 14 patients with PNH, from 58 healthy volunteers, and from 27 patients with aplastic anemia (AA). PB samples from 17 patients with PNH, from 14 normal volunteers, and from 37 patients with AA were analyzed. The diagnosis of PNH was established by clinical ®ndings and results of the Ham test (Table 1; laboratory parameters correspond to the time of sampling). The PNH group consisted of patients with predominantly hemolytic disease, cases of PNH/AA syndrome, MDS, or acute myelogenous leukemia. Patients with hemolytic PNH did not have a previous history of AA, lacked signi®cant neutropenia or thrombocytopenia, and had normocellular or hypercellular BM morphology. The diagnosis of AA was established according to the BM biopsy specimen and PB count criteria of the International Study of Aplastic Anemia and Agranulocytosis; severity was classi®ed by the criteria of Camitta et al.21 Patients with MDS were classi®ed according to the French-American-British Classi®cation criteria.22 We analyzed PB and BM samples from AA patients after hematopoietic recovery, (Pharmingen, San Diego, CA). Platelets were identi®ed by counterstaining with FITC-conjugated CD41a or PE-conjugated CD42b MoAb (Becton Dickinson) and were stained to estimate the expres-

Table 1. Clinical Characteristics of PNH Patients

<table>
<thead>
<tr>
<th>ID</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Duration (yr)*</th>
<th>Hemoglobin (mg/dL)</th>
<th>ANC (per μL)</th>
<th>Plt (&lt;10^12/μL)</th>
<th>Ham’s %</th>
<th>BM Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>M</td>
<td>AA</td>
<td>9</td>
<td>7.9</td>
<td>730</td>
<td>7</td>
<td>2</td>
<td>Normo</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>F</td>
<td>MDS</td>
<td>6</td>
<td>9.1</td>
<td>990</td>
<td>26</td>
<td>20</td>
<td>Hypo</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>F</td>
<td>AA</td>
<td>5</td>
<td>5.5</td>
<td>250</td>
<td>29</td>
<td>58</td>
<td>Hypo</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>M</td>
<td>AA</td>
<td>18</td>
<td>8.7</td>
<td>2,500</td>
<td>8</td>
<td>20</td>
<td>Hypo</td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>M</td>
<td>MDS</td>
<td>2</td>
<td>10.3</td>
<td>1,500</td>
<td>67</td>
<td>33</td>
<td>Hypo</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>F</td>
<td>AA</td>
<td>3</td>
<td>11</td>
<td>3,000</td>
<td>57</td>
<td>11</td>
<td>Hypo</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>M</td>
<td>AA</td>
<td>4</td>
<td>7.1</td>
<td>2,500</td>
<td>48</td>
<td>23</td>
<td>Hypo</td>
</tr>
<tr>
<td>8</td>
<td>30</td>
<td>F</td>
<td>AA</td>
<td>7</td>
<td>10.5</td>
<td>1,900</td>
<td>120</td>
<td>17</td>
<td>Normo</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>M</td>
<td>AA</td>
<td>1</td>
<td>9.7</td>
<td>3,700</td>
<td>25</td>
<td>3</td>
<td>Normo</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>F</td>
<td>AA</td>
<td>7</td>
<td>14</td>
<td>4,200</td>
<td>88</td>
<td>2</td>
<td>Hypo</td>
</tr>
<tr>
<td>11</td>
<td>34</td>
<td>F</td>
<td>AML</td>
<td>11</td>
<td>6.2</td>
<td>20</td>
<td>7</td>
<td>2</td>
<td>Hypo</td>
</tr>
<tr>
<td>12</td>
<td>43</td>
<td>F</td>
<td>Hemol</td>
<td>4</td>
<td>9</td>
<td>3,500</td>
<td>125</td>
<td>43</td>
<td>Hypo</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>F</td>
<td>AA</td>
<td>3</td>
<td>12.8</td>
<td>690</td>
<td>154</td>
<td>7.3</td>
<td>Hypo</td>
</tr>
<tr>
<td>14</td>
<td>16</td>
<td>F</td>
<td>AA</td>
<td>6</td>
<td>12.3</td>
<td>3,540</td>
<td>141</td>
<td>80</td>
<td>Normo</td>
</tr>
<tr>
<td>15</td>
<td>53</td>
<td>F</td>
<td>AA</td>
<td>6</td>
<td>9.2</td>
<td>2,800</td>
<td>23</td>
<td>3</td>
<td>Normo</td>
</tr>
<tr>
<td>16</td>
<td>30</td>
<td>M</td>
<td>Hemol</td>
<td>1</td>
<td>7.7</td>
<td>1,610</td>
<td>30</td>
<td>14</td>
<td>Normo</td>
</tr>
<tr>
<td>17</td>
<td>29</td>
<td>M</td>
<td>Hemol</td>
<td>10</td>
<td>9.3</td>
<td>1,710</td>
<td>142</td>
<td>38</td>
<td>Normo</td>
</tr>
</tbody>
</table>

* Time from the initial diagnosis.

**Flow cytometric analysis.** Phycocyanin (PE)-conjugated HPCA-2 MoAb directed against human CD34 antigen (Becton Dickinson, Mountain View, CA) was used for the quantitation of CD34+ cells in PB and BM according to the modification of the procedure described by Sutherland et al.23 Briefly, 100 μL of PB was stained for 30 minutes with appropriate MoAb or isotype-matched control IgG (Becton Dickinson), lysed, and fixed using Q-prep reagent (Coulter, Miami, FL). Samples were analyzed using a flow cytometer (Epics ELITE; Coulter). Depending on the cellularity of the sample, variable numbers of cells were analyzed until 100 to 1,000 positive events were collected in a gate set using isotopic controls. The frequency of CD34+ cells in BM was measured after isolation of BMMNCs. BM cells were incubated with appropriate directly conjugated MoAb and analyzed in a similar fashion as that described for PB. The frequency of CD34+ cells was calculated by dividing the number of CD34+ cells by the total number of cells analyzed and expressed as per 10^5 MNCs for BM and per 10^5 PBMCs or per milliliter of PB. For the measurement of the expression of GPI-anchored molecules, a panel of MoAbs was used. PE-labeled antihuman CD59 (Research Diagnostics Inc, Flanders, NJ) was combined with fluorescein isothiocyanate (FITC)-conjugated HPCA-1 anti-CD34 MoAb. In some experiments Cy-PE-labeled CD34 MoAb (Immunotech, Westbrook, ME) was used in conjunction with PE-labeled antihuman CD59 MoAb and FITC-labeled CD55 MoAb (Pharmingen, San Diego, CA). Platelets were identi®ed by counterstaining with FITC-conjugated CD41a or PE-conjugated CD42b MoAb (Becton Dickinson) and were stained to estimate the expres-

**BM cell preparation.** BM was obtained by aspiration from the posterior iliac crest into syringes containing media supplemented 1:10 with heparin (O’Neill and Feldman, St Louis, MO). Informed consent was obtained according to a protocol approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. Mononuclear cells (MNCs) from BM and PB were isolated by density gradient centrifugation using lymphocyte separation medium (Organon, Durham, NC). After washing in Hank’s balanced salt solution (Life Technologies, Gaithersburg, MD), cells were resuspended in Iscove’s modi®ed Dulbecco’s medium (Life Technologies) supplemented with 20% fetal calf serum (Life Technologies).
MoAb for monocytes and PE-conjugated CD59 MoAb and FITC-conjugated CD16 MoAb for granulocytes. In all staining procedures PE-, Cy-PE, or FITC-conjugated mouse isotype-matched MoAbs served as controls. In PB samples, using the monocyte or granulocyte gate, the number of cells showing both CD14 and CD13 (monocyte gate) or CD16 and CD15 (granulocyte gate) was divided by the total number of CD13 or CD15 cells in the gate and expressed as percentage. Similar methods were used to estimate the percentage of CD59+ platelets within the CD42b+ patient population and to calculate the percentage of CD59+/CD55+ CD34+ cells. All data were analyzed using EpilInfo, Version 5 software (Centers for Disease Control, Atlanta, GA).

Separation of CD34+ cells. After washing with phosphate-buffered saline (PBS; Life Technologies) supplemented with 2% human albumin, cells were labeled by biotinylated antihuman CD34 MoAb (Cellpro, Bathell, WA) and were applied to an affinity column containing biotin-coated beads, and the CD34+ cell fraction was eluted with PBS. An aliquot of the eluted cells was stained with PE-conjugated CD34 HPACA-2 MoAb (Becton Dickinson) to assess the purity of the eluted cells. Usually, 70% to 90% of separated cells were CD34+. For higher purity preparations, cells were further fractionated; column-purified cells were stained with FITC-labeled CD34+ MoAb, washed with PBS, and sorted by microcytometry (Epics ELITE; Coulter). The purity of cells obtained by combining affinity chromatography and flow cytometry was 97% to 99%. For sorting based on the expression of CD59 antigen, CD34 affinity column-purified CD34+ cells or BMMNCs were stained with PE-conjugated antihuman CD34 MoAb and FITC-conjugated anti-CD34 MoAb and sorted in the blast gate as CD34+CD59+ and CD34+CD59- cells. The viability of sorted cells was 85% to 95%. The numbers of sorted cells were adjusted for the viability.

Hematopoietic cell culture. Hematopoietic colony-forming cells (CFCs) in BM and PB of AA patients were measured in methylcellulose cultures under standardized conditions. Freshly isolated BMMNCs were plated in methylcellulose (Stem Cell Technologies, Vancouver, Canada) in the presence of 50 ng/mL interleukin-3, 20 ng/mL granulocyte-macrophage colony-stimulating factor, 50 ng/mL stem cell factor, and 2 U/mL erythropoietin (Amgen, Thousand Oaks, CA). All factors from BMMNCs from normal volunteers were plated at a density of 1 × 105, and those from AA patients were plated at the density of 3 × 105 cells in 1 mL culture medium in 35-mm dishes. CD34+ cells were plated at a density of 1 × 105 cells/0.5 mL methylcellulose in 48-well, 11-mm plates. Primary methylcellulose cultures of affinity column-purified CD34+ cells or further fractionated cells (CD34+CD59+ and CD34+CD59- cells) were performed as described above for total BMMNCs.

LTBMCs for the determination of LTC-ICs were performed according to modifications of published methods. Allogeneic stroma derived from 10 × 106 BMMNCs was grown to confluence in stem cell media (Stem Cell Technologies) supplemented with 1 × 10−4 mol/L hydrocortisone 21-hemisuccinate (Sigma, St Louis, MO), which was replaced weekly. After 3 weeks, stromal cells were trypsinized, washed, and placed in 48-well plates. After reestablishment of confluent cellular layers, the plates were irradiated (15 Gy of 250-KV x-rays) and used for the measurement of LTC-IC content in MNC fractions obtained from BM and PB. To assure consistency of results in all experiments, stroma from only 5 normal donors was used. These stromal preparations showed comparable feeder function as measured by the number of LTC-ICs in healthy volunteers. At least 2 (3 if cellularity of the sample was sufficient) cell concentrations per sample were placed on the preestablished, irradiated stromal feeder layers and cultured at 33°C for 5 weeks. Media changes were performed weekly. After 5 weeks of culture, adherent cells were harvested using trypsin (Life Technologies), washed, and replated in duplicate in methylcellulose to estimate the numbers of cells forming secondary colonies. Where possible, to convert the numbers of secondary CFCs observed at 5 weeks to LTC-ICs, limiting dilution experiments were performed to quantitate colonies derived from a single LTC-IC. Decreasing numbers of BMMNCs and PBMCs or purified CD34+ cells were plated in 96-well flat-bottom plates, containing preformed irradiated allogeneic stroma. For each dilution, 12 or 24 repetitive wells were seeded. After 5 weeks of culture under previously described conditions, cells from each well were trypsinized and transfected into methylcellulose supplemented with growth factors. After an additional 2 weeks, wells containing colonies were scored. Using limiting dilution, the frequency of LTC-ICs and the mean number of colonies per positive well (per LTC-IC; clonogenic capacity of individual LTC-ICs) were calculated. The clonogenic capacity of a single LTC-IC could be further calculated by dividing the number of colonies derived from bulk cultures by the frequency of LTC-ICs. Because limiting dilutions experiments were only possible for some PNH and AA patients, the number of clonogenic cells was converted to the absolute number of LTC-ICs by dividing the numbers of colonies in bulk cultures by the mean clonogenic capacity of LTICs measured in limiting dilution experiments. Based on these numbers, the frequency of LTC-ICs per 105 cells or per microliter of PB was then calculated.

Statistical analysis. A nonparametric Kruskal-Wallis Test was used to evaluate statistical differences in the number of CFCs, CD34+ cells, and LTC-ICs within groups. Correlation between levels of GDF-deficiency within the CD34+ cell population, monocytes, erythrocytes, and granulocytes was assessed using linear regression analysis.

RESULTS

Frequency of CD34+ cells, CFCs, and LTC-ICs. PNH is often associated with BM failure. We tested whether the numbers of progenitors and stem cells are decreased in patients with PNH, with or without underlying BM disorder such as AA and MDS (Table 1). Expression of CD34 antigen can be used to quantify immature hematopoietic cells by flow cytometry. Significantly decreased frequencies of CD34+ cells were found in the BM and PB of patients with PNH (Table 2). There was no significant difference between the numbers of CD34+ cells in patients with predominantly hemolytic PNH compared with those in PNH with associated AA or MDS. As assessed functionally in colony assays,

Table 2. Frequency of CD34+ Cells, CFCs, and LTC-ICs in Patients With PNH

<table>
<thead>
<tr>
<th></th>
<th>BM CFCs</th>
<th>LTC-ICs</th>
<th></th>
<th>BM CFCs</th>
<th>LTC-ICs</th>
<th></th>
<th>PB CFCs</th>
<th>LTC-ICs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal volunteers</td>
<td>2,982 ± 202 (n = 58)</td>
<td>211 ± 124 (n = 38)</td>
<td>1.97 ± 0.4 (n = 10)</td>
<td>5,867 ± 602 (n = 14)</td>
<td>5 ± 0.8 (n = 14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNH patients</td>
<td>627 ± 159 (n = 11)</td>
<td>62 ± 27 (n = 5)</td>
<td>0.24 ± 0.096 (n = 7)</td>
<td>801 ± 222 (n = 14)</td>
<td>0.1 ± 0.04 (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In BM, the values are expressed as mean ± SEM of CD34+, CFC, and LTC-IC numbers per 10⁶ MNCs. In PB, the values represent mean ± SEM of CD34+ and LTC-IC numbers per microliter of blood.
committed progenitors in PNH BM were similarly decreased (Table 2). We measured LTC-ICs as a surrogate for primitive hematopoietic cells. LTC-ICs in PNH BM and PB also were drastically reduced in comparison with that in normal BM and PB. The decrease in the number of LTC-ICs was more pronounced than the changes in the numbers of CD34⁺ cells and CFCs. We calculated that the long-term culture-initiating capacity of PNH CD34⁺ was markedly reduced, and the moderate decrease in the CD34 cell number cannot account for the drastic reduction in the numbers of LTC-ICs (Table 2).

Expression on GPI-linked proteins on CD34⁺ cells and mature BM cells. We sought a suitable marker for GPI-anchored protein deficiency on hematopoietic progenitors by first determining the expression of different GPI-linked proteins on CD34⁺ cells from normal donors. Most of the tested antigens were expressed on only a proportion of CD34⁺ cells: CD24 on 60% ± 12% (mean ± SD), CDw54 on 60% ± 10%, CD58 (LFA-3) on 89% ± 14%, and CDw90 (Thy-1) on 91% ± 14% of CD34⁺ cells (data not shown). Therefore, we did not consider any of these antigens to be suitable markers for GPI-deficient CD34⁺ cells. In contrast, CD55 and CD59 antigens were expressed on at least 99% of normal CD34⁺ cells (Figs 1 and 2A and B). Three-color flow cytometric analysis using CD55, CD59, and CD34 MoAbs also showed that the great majority of CD34⁺ cells simultaneously expressed CD55 and CD59 antigens; because the expression of CD59 was more homogeneous (Fig 1A and 1B), we selected CD59 for subsequent analysis of CD34⁺ cells from patients with AA and PNH. To confirm that CD59 was present on the cell surface membrane in a GPI-anchored form, cells were exposed to enzymatic digestion with phospholipase C (PPLC; 1 U/mL). PPLC hydrolysis of GPI-anchor and subsequent flow cytometric analysis of CD59 expression on CD34⁺ cells showed 0.5-log shift in fluorescence intensity after 20 minutes (Fig 1D).

Using CD59 antigen as a parameter for GPI-anchored protein deficiency, we observed that greater than 99.9% of CD34⁺ cells in normal BM expressed this protein (Table 3). In 12 PNH patients with a positive Ham’s test, 28% ± 8.2% of CD59⁻ cells were detected within the CD34⁺ cell population. In contrast, GPI-deficient (CD59⁻) CD34⁺ cells were present in 6 of 37 patients with AA (Figs 2 and 3). For comparison, in parallel experiments, we also determined expression of GPI-anchored proteins on PB monocytes, platelets, and neutrophils. Expression of CD14 on CD13⁻ mono-
cells, CD16 on CD15+ neutrophils, and CD59 on CD41a+ platelets were used as parameters for GPI deficiency in these hematopoietic lineages. When GPI-anchored proteins were used as a marker, about 10% of PB cells from normal donors failed to express a lineage-specific GPI-anchored antigen, implying that lack of multiple GPI-linked membrane proteins needs to be identified to increase the specificity of the assay (Table 3). PNH patients showed significantly higher percentages of GPI-deficient cells, with 77% ± 8% monocytes, 53% ± 9% neutrophils, and 42% ± 6% platelets failing to react with MoAbs directed against GPI-anchored molecules. There was no statistically significant correlation between GPI-anchored protein deficiency within the CD34+ cell population and low expression of lineage-appropriate proteins of this class on neutrophils, monocytes, platelets, or erythrocytes.

To avoid false-negative results, we also used a combination of two MoAbs directed against GPI-anchored molecules. By this method, the specificity of staining in PB was increased, so that 99.9% to 100% of normal monocytes (CD13+) expressed either CD59, CD14, or both antigens and 100% neutrophils (CD15+) expressed either CD59, CD16, or both antigens (data not shown). When this technique was applied to PB cells from PNH patients, the numbers of GPI-deficient monocytes, granulocytes, and platelets decreased by an average 10% (data not shown).

Cloning efficiency of CD34+ cells from PNH patients. The numbers of CD34+ cells, CFCs and LTC-ICs appeared to be reduced in patients with PNH. Therefore, we directly tested CD34+ cells derived from PNH for their colony-forming ability in primary methylcellulose cultures and for their long-term culture-initiating capacity. Purified CD34+ cells from 4 patients with PNH showed poor primary colony formation as compared with that of CD34+ cells from normal donors (Fig 4). To measure whether decreased colony formation was caused by defective proliferation of the PIG-A- deficient CD34+ cells, CD34+ cells from these patients were sorted based on expression of CD59 antigen, and colony formation was compared for CD34+ CD59+ and CD34+ CD59− cells. Both cell populations (CD34+ CD59+ and CD34+ CD59− cells) showed similarly low colony-forming ability (in comparison with that of normal CD34+ cells; see Fig 5). The content of LTC-ICs within these two CD34+ cell subpopulations was also decreased (Fig 6).

Phenotype of progeny derived from CD34+ cells from patients with PNH. To determine whether progeny cells produced in vitro by CD34+ CD59− cells were deficient for GPI-anchored proteins, we measured CD59 expression on cells derived from CD34+ cells grown in vitro. For colonies derived from CD34+ cells of PNH patients, a significant

**Table 3. Expression of GPI-Linked Proteins on Blood Cells Derived From Patients With PNH**

<table>
<thead>
<tr>
<th></th>
<th>CD34+CD59 in % of CD34+ Progenitor Cells</th>
<th>CD14+CD13 in % of CD14+ Monocytes</th>
<th>CD51αCD16 in % of CD16+ Granulocytes</th>
<th>CD41aCD59 in % of CD41a+ Plt</th>
<th>Ham’s Test Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal volunteers</td>
<td>1 ± 0.1 (n = 35)</td>
<td>17 ± 5 (n = 10)</td>
<td>10 ± 8 (n = 10)</td>
<td>11 ± 5 (n = 10)</td>
<td>ND</td>
</tr>
<tr>
<td>PNH patients</td>
<td>28 ± 8.2 (n = 9)</td>
<td>77 ± 8 (n = 12)</td>
<td>53 ± 9 (n = 12)</td>
<td>42 ± 6.7 (n = 12)</td>
<td>23 ± 3.5 (n = 12)</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM of cells lacking the expression of the antigens as indicated.
Abbreviations: Plt, platelets; ND, not done.
percentage of cells lacked CD59 expression, higher than the proportion of initial CD34+CD59- cells (Fig 4).

Normal BM CD34+ cells did not produce CD59+ progeny in methycellulose culture. When progeny derived from cultures of CD34+CD59+ and CD34+CD59- cells obtained from a single patient were tested for CD59 expression, only CD34+CD59- cells were observed to produce GPI-deficient progeny (as measured by CD59 expression), whereas the progeny of CD34+CD59- cells expressed normal levels of CD59 antigen (Fig 5). In some normal BM samples, a minor proportion of CD34+ cells lacked CD59 expression (<0.01%). We purified 400 of these cells and cultured them in methycellulose; CD34+CD59- cells showed similar colony formation to that of CD34+CD59+ cells derived from the same person, and both CD34+CD59- and CD34+CD59+ -derived progeny expressed CD59 antigen. However, comparison of CD59 fluorescence intensity levels showed that CD34+CD59- -derived progeny expressed lower levels of CD59 than did those derived from CD34+CD59+ cells (Fig 7). Thus, it is possible either that CD59 can be differentially expressed on more mature cells or that a small population of CD34+ cells with PIG-A mutation exists in some normal donors.

DISCUSSION

PNH, a disorder frequently observed in association with AA, arises at the level of the stem cell. Previously, investigators have inferred from clinical data that BM cells with the PNH phenotype had a survival advantage over cells with normal GPI-linked proteins and that this advantage was responsible for the association of PNH with AA. In assaying the number and function of hematopoietic progenitor and stem cells in patients with PNH, we addressed several issues relevant to the pathogenesis of this syndrome, especially in its relationship to BM failure. We found decreased numbers of immature hematopoietic progenitor cells, phenotypically defined by CD34 antigen expression, and decreased numbers of committed and primitive hematopoietic cells, measured by short- and long-term colony cultures, respectively. Purified CD34+ cells from PNH patients showed decreased clonogenicity compared with that of normal donors, suggesting a qualitative abnormality of the hematopoietic progenitor cell compartment in PNH. These findings extend previous observations by ourselves and others showing that hematopoietic CFCs of PB and BM are numerically decreased in both hemolytic and aplastic types of PNH, disproportionately to the abnormalities in BM morphology.
or PB counts. The reductions in both CD34+ cell and LTC-IC numbers were similar to those observed in AA uncomplicated by PNH. The proportion of GPI-anchored protein-deficient CD34+ cells in PB was lower than the percentages of mature cells with the PNH phenotype, a result consistent with either an intrinsically higher rate of proliferation for the abnormal cells or in vivo selection of GPI-anchored protein-deficient cells.

Although the poor proliferative capacity of PNH BM and PB in progenitor assays has been established, in the current study we further investigated the relative growth of normal and PNH cells from individual patients. In vitro, colony formation in methylcellulose and proliferation in LTBM C of fractions of GPI-deficient (CD59-) and normal (CD59+) CD34+ cells derived from the same donor were equivalent, suggesting that PIG-A-deficient cells do not have an innate growth advantage, at least as assessed in these systems. In vivo, the presence of selective factors favoring proliferation of GPI-deficient cells may lead to the expansion of PNH clones. Increased compensatory rates of erythropoiesis and megakaryocytopenia because of complement-mediated lysis of erythroid precursors and megakaryocytes has been postulated as one mechanism leading to BM failure in PNH.10,31 However, the finding of markedly decreased colony formation by PNH progenitor cells in the absence of activated complement argues against this theory and in support of an underlying proliferative defect common to both the abnormal and normal cells in these patients. Although there appears to be no growth disadvantage of CD59- progenitor cells as compared with CD59+ cells in patients with BM failure syndromes, this growth potential is substantially decreased compared with that in CD59+ progenitor cells from normal BM. The absence of a relative growth advantage of the CD59- progenitor cells may explain why cells with the PNH phenotype expand only in the context of BM failure. Markedly impaired hematopoiesis observed in laboratory assays in PNH helps explain the close association between this disease and other BM failure syndromes, including AA and MDS.8,9,18,19,32,35-37 We also examined the PB and BM of individuals with AA for evidence of PNH using our flow cytometric techniques. Analysis of BM appears to be more sensitive than analysis of PB for the early diagnosis of the PNH syndrome complicating AA.38 In our study, we used stringent phenotypic criteria for examination of GPI-anchored protein expression on CD34+ cells and detected GPI-deficient CD34+ cells in only 16% of new AA cases (n = 37) and in none of the MDS cases (n = 9). We used the CD59 antigen on CD34+ cells as a marker for the PNH phenotype because the expression of this surface antigen was more homogenous than that of the CD55 antigen. Although PNH has been postulated as one mechanism leading to BM failure in PNH.30,31 However, the finding of markedly decreased colony formation by PNH progenitor cells in the absence of activated complement argues against this theory and in support of an underlying proliferative defect common to both the abnormal and normal cells in these patients. Although there appears to be no growth disadvantage of CD59- progenitor cells as compared with CD59+ cells in patients with BM failure syndromes, this growth potential is substantially decreased compared with that in CD59+ progenitor cells from normal BM. The absence of a relative growth advantage of the CD59+ progenitor cells may explain why cells with the PNH phenotype expand only in the context of BM failure. Markedly impaired hematopoiesis observed in laboratory assays in PNH helps explain the close association between this disease and other BM failure syndromes, including AA and MDS.8,9,18,19,32,35-37 We also examined the PB and BM of individuals with AA for evidence of PNH using our flow cytometric techniques. Analysis of BM appears to be more sensitive than analysis of PB for the early diagnosis of the PNH syndrome complicating AA.38 In our study, we used stringent phenotypic criteria for examination of GPI-anchored protein expression on CD34+ cells and detected GPI-deficient CD34+ cells in only 16% of new AA cases (n = 37) and in none of the MDS cases (n = 9). We used the CD59 antigen on CD34+ cells as a marker for the PNH phenotype because the expression of this surface antigen was more homogenous than that of the CD55 antigen. Although previous studies have shown increased expression of CD59 and CD55 during differentiation,8,39 we found that CD59 was expressed on virtually all CD34+ cells from normal BM (in contrast to CD29, CD58, and CDw90). The use of an additional marker for GPI-deficiency for the phenotyping of CD34+ cells did not increase the sensitivity of flow cytometry in the diagnosis of PNH. That a PIG-A genotype in CD34+ cells was responsible for the absence of CD59 antigen expression (as opposed to selective downregulation of CD59) could be inferred from failure to detect both CD59 and CD55 expression on progeny from CD59- CD34+ cells.

Flow cytometric determination of GPI-anchored proteins on hematopoietic cells can be used to diagnose PNH.32,40-42 In our experience, when the absence of a single GPI-anchored antigenic marker was used as a diagnostic parameter, a significant proportion of negative cells was also observed in normal blood, obscuring the distinction between normal and PNH individuals. Analysis can be much improved when two antigens for each lineage are assessed, and there was good correlation between the absence of two constitutively expressed GPI-anchored markers and the presence of clinically symptomatic PNH (or a positive result from a Ham test). Using flow cytometry, blood from a high percentage of German AA patients was found to contain cells with PNH phenotype.13 The diagnosis of PNH in the German study was based on an abnormal ratio of negative cells in each lineage compared with that in normal lineages. Our lower proportion of PNH in AA was likely the result of differences in the method of determining a significant GPI-anchored protein-deficient population. The downregulation of expression of some of these proteins in the absence of a PIG-A lesion is also possible. Finally, we doubt but cannot exclude biological differences in European and American patient populations.

Although very small numbers of CD34+ cells with apparently absent CD59 expression (a phenotype suggestive of GPI deficiency) were found in a minor proportion of normal donors, the progeny of these cells showed decreased but not absent CD59 expression. Too few cells of the CD34+ CD59- phenotype could be isolated by the cell-sorting technique to allow for molecular analysis of the PIG-A gene. However,
when PNH clones arose in lymphoma patients treated with CAMPATH-1, an antibody that recognizes a GPI-anchored protein on lymphocytes, these cells were shown to be PIG-A-. Either these clones have a growth advantage in the failed BM, or they are less susceptible to BM-damaging agents.

In summary, analysis of hematopoietic stem and progenitor cell compartment in PNH implies that GPI-deficient clones may expand in the context of oligoclonal hematopoiesis when the chances of clonal recruitment of the PNH stem cell are higher. Our results do not favor an intrinsic proliferative advantage for PNH clones as measured in conventional assays of hematopoiesis.

REFERENCES

25. Shichishima T, Terasawa T, Saitoh Y, Hashimoto C, Ohto H, Maruyama Y: Diagnosis of paroxysmal nocturnal hemoglobinuria by phenotypic analysis of erythrocytes using two-colour flow cytometry with monoclonal antibodies to DAF and CD59/MACIF.


Impaired Hematopoiesis in Paroxysmal Nocturnal Hemoglobinuria/Aplastic Anemia Is Not Associated With a Selective Proliferative Defect in the Glycosylphosphatidylinositol-Anchored Protein-Deficient Clone

Jaroslaw P. Maciejewski, Elaine M. Sloand, Tadatsugu Sato, Stacie Anderson and Neal S. Young