Peripheral Blood Harvest of Unaffected CD34+ CD38- Hematopoietic Precursors in Paroxysmal Nocturnal Hemoglobinuria

By Gregory M. Prince, Mai Nguyen, Hillard M. Lazarus, Robert A. Brodsky, Leon W.M.M. Terstappen, and M. Edward Medof

Paroxysmal nocturnal hemoglobinuria (PNH) arises from somatic mutation of a bone marrow progenitor that disrupts glycosylphosphatidylinositolanchoring (GPI-anchoring) of cell surface proteins. We recently characterized the expression of GPI-anchored decay accelerating factor (DAF) and CD59 during hematopoietic development in PNH marrow. We found that, although a subset of early hematopoietic precursors identified by the CD34-CD38- phenotype exhibits normal DAF and CD59 expression, DAF and CD59 are absent on the majority of CD34+CD38- cells. Pluripotent CD34+CD38- hematopoietic stem cells normally circulate in the peripheral blood and can be collected by apheresis, cryopreserved, and later used for reconstitution of hematopoiesis. In this study, we examined the phenotypes of CD34+ cells that are released into the blood of PNH patients. Analyses of apheresis samples from three affected individuals showed discrete populations of circulating DAF-CD59+CD34+ and DAF-CD59-CD34+ cells. Variable proportions of CD34+CD38- cells were present within the peripheral blood CD34+ cells of each patient, but in all three cases the DAF-CD59+CD34+CD38- cell subset was enriched relative to the DAF-CD59-CD34+CD38- cell subset. Because CD34+ cells lacking CD38 antigen are highly enriched for self-renewing hematopoietic stem cells, these findings indicate that apheresis samples can serve as a source of unaffected stem cells for autologous marrow transplantation of PNH patients.

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Materials and Methods

Proteins. Murine anti-DAF and anti-CD59 monoclonal antibodies (MoAbs) IA10 and IF5 were obtained as described. Murine anti-CD34 and CD38 MoAbs were prepared by B. Abrahams at Becton Dickinson (San Jose, CA). Biotinylated anti-DAF-Rlpeptide (DAF-PE), CD59-PE, CD34-fluorescein isothiocyanate (FITC), CD34-pericinin chlorophyll A protein (PerCP), and...
CD38-allophycocyanin (APC) were prepared as previously described.\textsuperscript{13,14}

Patients. All PNH patients who consented to cytapheresis were studied. Patients A, B, and C had characteristic clinical manifestations and laboratory findings (including positive Ham acid hemolysis tests) of PNH. PNH in patients A and B was diagnosed during the investigation of hemolytic anemia. In addition, patient A suffers from systemic lupus erythematosus. Patient C developed PNH in the aftermath of aplastic anemia treated with immunosuppression, developing a positive Ham test on routine screening several months after treatment. All three patients had mild anemia (hematocrit, 28\% to 32\%). Patient C had normal cellularity and patients A and B were hypercellular on bone marrow examination. No evidence of dysplasia was seen in any of the patients. In each case, DAF-CD59\textsuperscript{-} subpopulations of erythrocytes and large proportions of DAF-CD59\textsuperscript{+} polymorphonuclear cells (see Results) were present in the peripheral blood. Control subjects were healthy laboratory personnel or were patients undergoing autologous peripheral blood progenitor cell harvest for lymphoma or solid tumors who were in remission at the time of study.

Cytapheresis. Peripheral blood mononuclear cell harvests were collected using a COBE Spectra blood cell separator (Lakewood, CO).\textsuperscript{15} Control subjects in some cases received prior subcutaneous injections of recombinant granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to increase the stem cell yield. In some instances, mononuclear cells were cryopreserved in 10\% dimethyl sulfoxide (final concentration) using a controlled-rate liquid-nitrogen freezer and stored in liquid nitrogen. Thawed cells were washed three times with RPMI containing 0.2 mg/mL DNAase 1 (Sigma Chemical Co, St Louis, MO).

Cell preparation and flow cytometry. Before staining, cells were washed three times in phosphate-buffered saline (PBS) containing 1\% BSA/0.1\% NaN\textsubscript{3} and resuspended to 1 \times 10\textsuperscript{6}/mL. Resuspended cells (0.1 mL) were incubated for 15 minutes on ice with 20 \mu L each of the designated antibodies at titrated concentrations. In studies involving biotinylated anti-DAF MoAb, resuspended cells (25 \mu L) were further incubated for 15 minutes on ice with 20 \mu L of titrated concentrations of Streptavidin-PE. After washing two times, cells were resuspended to 1 mL in PBS containing 0.5\% paraformaldehyde.

Flow cytometric analyses were performed on a FACStar Plus (Becton Dickinson) or a modified FACScan, which is equipped with an Argon ion laser tuned at 488 nm and a 633-nm HeNe laser. The HeNe permits the use of APC as a fluorochrome. Correction for the cross-over of fluorescence between two or more detectors was performed as described.\textsuperscript{15,14} Light scatter and two or three fluorescence signals were determined for each cell.

Data acquisition was performed with the Lysis II software (Becton Dickinson). For each experiment, two data files were collected. For the first data file, 15,000 to 30,000 events were collected without the use of gates to determine the frequency of CD34\textsuperscript{+} cells. For the second data file, gates were used on both light-scatter parameters, and CD34\textsuperscript{+} fluorescence and the entire sample originally containing 10\textsuperscript{6} cells was collected. Data analysis was performed using the Lysis II or Paint-A-Gate Prosoftware (Becton Dickinson).

RESULTS

CD34\textsuperscript{+} cells circulate in the peripheral blood at low frequencies.\textsuperscript{16} To investigate whether these circulating CD34\textsuperscript{+} cells normally express DAF and CD59 homogeneously and whether they derive from affected or unaffected hematopoiesis in PNH,apheresis samples from controls and PNH patients were stained with CD34-FITC and DAF-PE. Flow cytometric analyses of harvests from two control subjects and one PNH patient stained with CD34-FITC and DAF-PE. The top two plots on the right giving data from the two control subjects show that the CD34 population homogeneously expresses DAF. In contrast, the bottom right-hand plot containing data from the PNH patient shows two populations of CD34\textsuperscript{+} cells: DAF\textsuperscript{+} and DAF\textsuperscript{-}.

![Flow cytometric analyses of cytapheresis samples from two control subjects and one PNH patient stained with DAF (CD55) biotin/streptavidin-PE and CD34-FITC. The left-hand plots show side scatter versus forward scatter with a gate set to enrich for CD34\textsuperscript{+} cells. The right-hand plots show staining of the cells for CD34 and DAF. The plots depict a small population of cells that are CD34\textsuperscript{+}. The top two plots on the right giving data from the two control subjects show that the CD34 population homogeneously expresses DAF. In contrast, the bottom right-hand plot containing data from the PNH patient shows two populations of CD34\textsuperscript{+} cells: DAF\textsuperscript{+} and DAF\textsuperscript{-}.](image-url)
formally DAF⁺. In contrast, in the PNH patient, two populations of peripheral blood CD34⁺ cells were observed: DAF⁺ (74% of CD34⁺ cells) and DAF⁻ (26% of CD34⁺ cells). Parallel studies performed with cryopreserved cells from the PNH patient and using CD59 staining as well as DAF staining (not shown) showed the same staining patterns with DAF and CD59 antibodies and the same proportions of unaffected and affected cells as measured with fresh cells, indicating that the affected DAF⁻ cells were GPI-anchor deficient and that the two populations present in PNH were stable to cryopreservation and thawing.

The majority of CD34⁺ progenitor cells are committed to one of the hematopoietic cell lineages. Pluripotent non-lineage committed progenitor cells are present in a subset of CD34 cells that lack CD38 antigen. Expression of DAF and CD59 by CD34⁺CD38⁻ and CD34⁺CD38⁺ cells in the apheresis sample from a control subject and from the above studied PNH patient are shown in Fig 2, A and E, and B and F, respectively. In the figure, only CD34⁺ cells are shown and the expression of CD38 is plotted versus the expression of DAF or CD59. Four phenotypes are distinguished: (1) CD34⁺CD38⁻DAF⁻CD59⁻ (upper gray dots), (2) CD34⁺CD38⁻DAF⁺CD59⁻ (lower gray dots), (3) CD34⁺CD38⁺DAF⁺CD59⁻ (upper black dots), and (4) CD34⁺CD38⁺DAF⁻CD59⁻ (lower black dots). As seen in the figure, in the case of the control subject, both committed CD34⁺CD38⁺ and uncommitted CD34⁺CD38⁻ cells expressed DAF and CD59 homogeneously. In contrast, in the PNH patient, cells lacking DAF and CD59 were observed in the most primitive CD34⁺CD38⁻ cell population. Unexpectedly, a higher proportion of unaffected DAF⁺CD59⁺CD34⁺CD38⁻ progenitor cells was seen as compared with affected DAF⁻CD59⁻CD34⁺CD38⁻ cells (see Table 1).

Flow cytometric analyses of peripheral blood harvests of two other PNH patients are shown in Fig 2, C and G, and D and H, respectively. As observed with the first patient, discrete populations of peripheral blood DAF⁺ CD59⁺ CD34⁺ and DAF⁻CD59⁻CD34⁺ stem cells were distinguishable in each case. Likewise, as noted in the first patient, the numbers of unaffected CD59⁺CD34⁺CD38⁻ cells (lower grey dots) were greater than the numbers of affected peripheral blood CD59⁻CD34⁺CD38⁻ cells (lower black dots) present in each patient. (Because of greater fluorescence intensity, CD59 is a more accurate marker than DAF for the assessment of the
presence of GPI-anchored proteins.) In Table 1 the percentages of peripheral blood CD34+ cells and the proportions of CD34+CD38- and CD34+CD38+ cells that expressed DAF and CD59 in the normal control and the three PNH apheresis samples are shown.

Analyses of a paired sample of BM available from PNH patient C are shown in Fig 3. As in Fig 2, only CD34+ cells are shown in the figure. The gray dots represent CD34+ cells that are DAF+ or CD59+, whereas the black dots represent cells that lack DAF or CD59. Comparison of the numbers of DAF-CD59- (black dots) and DAF+CD59+ (gray dots) cells showed that greater than 95% of the CD34+ cells in the marrow of this patient were affected. In the other two patients, staining of peripheral blood cells for DAF and CD59 (Fig 4) showed that in both cases, the great majority of neutrophils that have short half-times in the circulation and, thus, reflect marrow production were affected. In Table 2 the proportions of unaffected DAF+ and CD59+ neutrophils in each patient are shown in relation to the proportions of unaffected DAF+ and CD59+CD34+CD38- stem cells in the corresponding apheresis sample.

**DISCUSSION**

Previous studies of DAF and CD59 expression by hematopoietic precursors in normal BM showed that both proteins are expressed homogeneously on the earliest identifiable hematopoietic progenitors distinguished by the CD34+CD38- phenotype. That the CD34+CD38- cell subset contains the

### Table 1. Percent Affected and Unaffected Cells in CD34+ Subpopulations

<table>
<thead>
<tr>
<th></th>
<th>%CD34</th>
<th>CD38- (DAF-)</th>
<th>CD38+ (DAF+)</th>
<th>CD38- (CD59-)</th>
<th>CD38+ (CD59+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.1</td>
<td>77 (98)</td>
<td>23 (94)</td>
<td>78 (100)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>PNH A</td>
<td>0.6</td>
<td>19 (39)</td>
<td>81 (79)</td>
<td>14 (36)</td>
<td>86 (98)</td>
</tr>
<tr>
<td>PNH B</td>
<td>0.2</td>
<td>93 (6)</td>
<td>7 (40)</td>
<td>96 (22)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>PNH C</td>
<td>0.7</td>
<td>89 (10)</td>
<td>11 (23)</td>
<td>83 (11)</td>
<td>17 (51)</td>
</tr>
</tbody>
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%CD34, % of CD34+ cells in leukopheresis; CD38-, % of CD38- cells within the CD34+ cell population; DAF+, % within indicated fraction which expresses CD55; CD38+, % of CD38+ cells within the CD34+ cell population; CD59-, % within indicated fraction which expresses CD59.

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**Fig 3.** Dot plots of paired leukopheresed blood and BM samples from PNH patient C stained for CD34, CD38, and DAF or CD59. Only CD34+ cells are shown. CD34+DAF-CD59- (black dots) and CD34+DAF+CD59+ (gray dots) cells are seen in both the peripheral blood and the BM. The population of gray dots representing CD34+DAF- and CD34+CD59- cells contains CD38+ pluripotent progenitor cells. In this patient, this small subpopulation is enriched in the leukopheresed sample.
pluripotent stem cell population has been confirmed by the ability of single cells of this phenotype to give rise to both lymphoid and myeloid progeny in culture. Analyses of marrow from two PNH patients showed large populations of DAF'CD59'CD34'CD38' progenitors, with smaller but distinct populations of DAF'CD59'CD34'CD38' progenitors. Consistent with this finding, two distinct lines of hematopoiesis, one normal and the other abnormal, were observed with affected and unaffected precursors present along the differentiation pathways of the myeloid, erythroid, and B-lymphocyte lineages. The identification in PNH marrow of CD34'CD38' cells that are phenotypically normal has important therapeutic implications because these cells constitute a potential source of stem cells for autologous marrow transplantation. However, for such transplantation to be feasible, practical methods must be devised to harvest these cells and return them to patients.

The present study indicates that stem cells recovered from apheresis samples could be used as a source of progenitor cells for hematopoietic reconstitution in PNH. Indeed, it may be a desirable source because, compared with BM harvest, peripheral stem cell harvest is less traumatic and can be performed repeatedly without anesthesia. Although a paired BM sample was available for analysis in only one patient, it may be presumed that the proportion of affected stem cells in all three patients is high because greater than 90% of mature circulating neutrophils that have been considered an accurate marker of BM production were affected in the other two cases. Although cytopheresis samples could be obtained on only three patients, unexpectedly, we found that the CD34'CD38' cells present in the circulation of all three patients were predominantly CD59' (see Fig 3 and Table 2). Thus, our findings indicate that unaffected progenitors are efficiently released from the marrow in PNH patients and circulate normally as in healthy individuals. It is noteworthy that in one patient (patient A), analyses of circulating progenitors showed that greater than 70% (in two different studies) were unaffected, as compared with only 2% to 9% DAF'CD59' neutrophils.

The mechanism of the apparently greater representation of unaffected CD34'CD38' cells in the circulation of patients is unknown. One possibility is that one or more GPI-anchored proteins could play a role in adherence/homing reactions that are involved in stem cell transfer from the marrow to the circulation. A second possibility is that affected DAF'CD59'CD34' cells are somehow protected from complement in the marrow, but upon transfer to the blood, either are destroyed and/or eliminated, or are induced to differentiate as a result of autologous complement attack.

A number of questions remain before autologous hematopoietic reconstitution can be exploited clinically. First, purification methods, using anti-CD59 or anti-DAF affinity chromatography or possibly complement lysis, must be developed to selectively purify unaffected DAF'CD59'CD34' cells from stem cell harvests. Second, further questions must be answered regarding basic pathophysiologic mechanisms underlying PNH. Some evidence indicates that unaffected stem cells in PNH marrow grow at a slower rate than stem cells of normal individuals, an implication that the hematopoietic microenvironment in PNH patients may provide a proliferative disadvantage to unaffected progenitors. Support for this hypothesis comes from the fact that up to 25% of PNH cases evolve either from or into aplastic anemia. Other evidence suggests that the affected PNH clone may have a relative proliferative advantage such that it is able to expand despite the presence of much larger numbers of unaffected stem cells. In support of such an advantage, the growth of PNH marrow cells in the absence of stimulating growth factors has been reported.

Notwithstanding the above considerations, further studies need to be performed to make autologous transplantation feasible.

Table 2. Percent Unaffected Cells in Mature Peripheral Blood Neutrophils and in Early (CD34+ and CD38+) Precursors

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<tr>
<th></th>
<th>Neutrophils</th>
<th>CD34'CD38' Stem Cells</th>
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<tr>
<td></td>
<td>DAF' (%)</td>
<td>CD59' (%)</td>
</tr>
<tr>
<td>PNH A</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>PNH B</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>PNH C</td>
<td>4</td>
<td>3</td>
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feasible. It is now routine to administer colony-stimulating factors to patients before peripheral stem cell harvests to increase the yield of circulating progenitors. We were reluctant to do this in our PNH patients because of uncertainty as to its effect on the patients' abnormal clones. Confirmatory in vitro studies on the ability of the unaffected progenitors to give rise to normal progeny need to be performed. Finally, mechanisms to selectively purify unaffected DAF+CD59+ progenitors must be developed. Nonetheless, our results indicate that peripheral stem cell harvest may be an important first step in the isolation of unaffected hematopoietic stem cells if autologous BM reconstitution of PNH patients is undertaken.

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