Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria: Search for a Pathogenetic Link


The association of paroxysmal nocturnal hemoglobinuria (PNH) and aplastic anemia (AA) raises the yet unresolved questions as to whether these two disorders are different forms of the same disease. We compared two groups of patients with respect to cytogenetic features, glycosylphosphatidylinositol (GPI)-linked protein expression, protein C/ protein S/thrombomodulin/antithrombin III activity, and PIG-A gene expression. The first group consisted of eight patients with PNH (defined as positive Ham and sucrose tests at diagnosis), and the second, 37 patients with AA. Twelve patients with AA later developed a PNH clone. Monoclonal antibodies used to study GPI-linked protein expression (CD14 on monocytes, CD16 on neutrophils), CD48 on lymphocytes and monocytes, CD67 on neutrophils and eosinophils, and, more recently, CD55, CD58, and CD59 on erythrocytes were also tested on a cohort of 20 normal subjects and five patients with constitutional AA. Ham and sucrose tests were performed on the same day as flow-cytometric analysis. Six of 12 patients with AA, who secondarily developed a PNH clone, had clinical symptoms, while all eight patients with PNH had pancytopenia and/or thrombosis and/or hemolytic anemia. Cytogenetic features were normal in all but two patients. Proteins C and S, thrombomodulin, and antithrombin III levels were within the normal range in patients with PNH and in those with AA (with or without a PNH clone). In patients with PNH, CD16 and CD67 expression were deficient in 78% to 98% of the cells and CD14 in 76% to 100%. By comparison, a GPI-linked defect was detected in 13 patients with AA, affecting a mean of 32% and 33% of CD14/CD67 and CD14 cell populations, respectively. Two of three tested patients with PNH and 1 of 12 patients with AA had a defect in the CD48 lymphocyte population. In a follow-up study of our patient cohort, we used the GPI-linked molecules on granulocytes and monocytes investigated earlier and added the study of CD55, CD58, and CD59 on erythrocytes. Two patients with PNH and 14 with AA were studied for 6 to 13 months after the initial study. Among patients with AA, four in whom no GPI-anchoring defect was detected in the first study had no defect in follow-up studies of all blood-cell subsets (including erythrocytes). Analysis of granulocytes, monocytes, and erythrocytes was performed in 7 of 13 AA patients in whom affected monocytes and granulocytes were previously detected. A GPI-anchoring defect was detected on erythrocytes in five of six. However, in one patient, who had a GPI-anchoring defect on leukocytes, we did not observe a defect on erythrocytes. In three patients without a GPI-anchoring defect after the initial study, a defect was detected on leukocytes only, with persistent negative Ham and sucrose tests in one patient, in all cell subsets in another, and on erythrocytes only in the remaining patient. Finally, PIG-A gene expression, studied by Northern blot analysis, showed that the PIG-A full-length transcript was barely detectable or absent in four patients with PNH and abnormal in length in one. All nine patients with AA who secondarily developed a PNH clone had a normal-sized PIG-A gene transcript. In conclusion, this study failed to show any significant difference, except for the size of the PNH clone, between PNH arising in patients with previous AA and in patients with de novo PNH. © 1995 by The American Society of Hematology.

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AA patients who only secondarily developed PNH (negative Ham and sucrose tests at diagnosis) to search for a pathogenetic link between these two syndromes.

MATERIALS AND METHODS

Patients

Patients

Patients were aware of the objectives of the study, and specimens were collected in accordance with the requirements established by the local ethics committee of the Hôpital Saint-Louis. The patients' main characteristics are listed in Table 1.

Thirty-seven consecutive patients with acquired AA were included in this study. Among these 37 patients with negative Ham/sucrose tests at diagnosis, 31 patients had an idiopathic form of AA, three had drug-induced AA, two had AA associated with an autoimmune disease, and one had viral hepatitis before AA. Thirteen patients met the international criteria for the severity of the disease. These patients received cyclosporine (CSA) or antithymocyte globulin (ATG) plus prednisone, with or without androgens, as first-line therapy. As second-line therapy, 14 patients received either a second course of ATG, CSA, and/or recombinant human growth factor (injection). Blood samples collected on EDTA were tested by indirect immunofluorescence. All of these antibodies were used at saturating concentrations, previously determined by densitometry using Quantiscan software (Bio-Rad, Hercules, CA). Using forward and side-scatter gating, it was possible to analyze lymphocytes and monocytes separately from the peripheral blood mononuclear cell (PBMC) fraction. Granulocytes were separated from monocytes in whole-blood samples, and 10,000 cells were analyzed in each sample through a granulocyte live-scatter gate. A minimum of 1,000 events was analyzed in a monocyte gate. The mean fluorescence intensity (MFI) was expressed on a logarithmic scale.

Ham Acid Lysis and Sucrose Tests

Ham and sucrose tests were performed on the same day as part of the routine work-up according to Dacie and Lewis. As a rule, the results of the lytic tests are given in pluses rather than as a percentage of lysis in our institution. However, results were quantitated by spectrophotometry in 12 patients whose lytic tests and respective spectrometry results are used as references for the laboratory; Y. Bretagne, CTS Hôpital St Antoine, Paris, France, unpublished results). For the sucrose test, (+) corresponds to less than 5% lysis (2% to 5%), + to 10% lysis (8% to 15%), and ++ to greater than 30% lysis. For the Ham test, (+) corresponds to 8% lysis (8% to 12%), + to 15% lysis (10% to 20%), and ++ to greater than 30% lysis (25% to 40%).

Cytogenetic Studies

Cytogenetic studies were performed after bone marrow culture in vitro for 24 and/or 48 hours. RHG and GTG-bands were obtained as usual, and chromosomes were classified according to the international nomenclature.

Coagulation Tests

Proteins S and C-related antigens and thrombomodulin were measured using enzyme-linked immunosorbent assay (ELISA) methods. Proteins S and C and antithrombin III activity were measured using functional chromogenic techniques.

Study of PIG-A Gene Expression by Northern Blot Analysis

Total RNA was isolated from PBMC using the guanidine isothiocyanate method. Northern blots were prepared using formaldehyde-containing gels. The blots were probed with PIG-A cDNA after labeling by random priming (Amersham, Les Ulis, France; Megaprime DNA labeling system). The probe corresponds to the full-length PIG-A cDNA and was a generous gift of Dr J. Takeda. The same filters were subsequently hybridized with a β-actin probe and used as a control. A semiquantitative estimation of PIG-A expression was performed by densitometry using QuantiScan software (The Imager, Applied, Strasbourg, France).
Thrombosis.

Globulin; CSA, cyclosporine A; IL-3, interleukin-3; G-CSF, granulocyte colony-stimulating factor; andro, androgens; H, hemolysis; inf, infections.

Clinical Manifestations

Table 1. Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age (yr/sex)</th>
<th>Diagnosis</th>
<th>Interval Diagnosis/Study</th>
<th>Etiology</th>
<th>Treatment</th>
<th>Symptoms</th>
<th>Ham &amp; Sucrose Tests at Inclusion</th>
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<tr>
<td>1</td>
<td>43/F</td>
<td>SAA</td>
<td>4 yr</td>
<td>T</td>
<td>ATG/CSA/IL3</td>
<td>H</td>
<td>+/-</td>
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<td>I</td>
<td>ATG</td>
<td>H</td>
<td>+/-</td>
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<tr>
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<td>ATG/CSA/andro/IL3</td>
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<td>(+)/-</td>
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<td>3 yr</td>
<td>I</td>
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<td>(+)/-</td>
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<td>(+)/-</td>
</tr>
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<td>andro</td>
<td>H</td>
<td>+/-</td>
</tr>
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<td>I</td>
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<td>+/-</td>
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<tr>
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<td>ATG/CSA</td>
<td>H</td>
<td>+/+</td>
</tr>
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<td>(+)/-</td>
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<td>+/-</td>
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<tr>
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<td>AA</td>
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<td>I</td>
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<td>+/-</td>
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<td>+/-</td>
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<td>+/-</td>
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<td>+/-</td>
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<tr>
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<td>31/F</td>
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<td>I</td>
<td>predniione</td>
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<tr>
<td>32</td>
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<td>andro</td>
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<td>+/-</td>
</tr>
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<td>33</td>
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<td>T</td>
<td>CSA/andro</td>
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<td>I</td>
<td>ATG/CSA/andro/G-CSF</td>
<td>None</td>
<td>+/-</td>
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<tr>
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<td>53/F</td>
<td>PNH</td>
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<td>/</td>
<td>ATG/CSA</td>
<td>H-inf-AA</td>
<td>+/-</td>
</tr>
<tr>
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<td>/</td>
<td>andro</td>
<td>Th-H-AA</td>
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<tr>
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<td>PNH</td>
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<td>/</td>
<td>ATG/CSA</td>
<td>Th-H-AA</td>
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<td>/</td>
<td>andro</td>
<td>H</td>
<td>+/-</td>
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<tr>
<td>42</td>
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<td>PNH</td>
<td>2 yr</td>
<td>/</td>
<td>/</td>
<td>Th-H</td>
<td>+/-</td>
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<td>/</td>
<td>/</td>
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<td>+/-</td>
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<td>/</td>
<td>/</td>
<td>AA</td>
<td>+/-</td>
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<td>PNH</td>
<td>1 mo</td>
<td>/</td>
<td>/</td>
<td>AA-H</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; SAA, severe AA; T, toxic; I, idiopathic, ai, autoimmune; post-hep, posthepatitis; ATG, antithymocyte globulin; CSA, cyclosporine A; IL-3, interleukin-3; G-CSF, granulocyte colony-stimulating factor; andro, androgens; H, hemolysis; inf, infections; Th, thrombosis.

RESULTS

Clinical Manifestations

Twelve of 37 patients with acquired AA secondarily developed PNH (as defined earlier), with a mean time from diagnosis to positive Ham and sucrose tests of 7.25 months (range, 5 to 19). None of these 37 patients were on therapy during the study. This complication was asymptomatic in six, whereas five patients had intravascular hemolysis and one had both intravascular hemolysis and recurrent infections. None had thrombosis. Among eight patients with PNH, pancytopenia was present at the time of the analysis in six, hemolytic anemia in six, and thrombosis (Budd Chiari syndrome) in three (Table 1).
Analysis of Monocytes

Monocytes were tested for CD14 GPI-linked antigen expression. Monocytes were recognized because of their light-scatter pattern and by CD13/CD64 double-staining. On average, 93.9% of monocytes from 20 normal donors and five patients with congenial AA expressed CD14, and all of these cells were positive for CD13 and CD64. In all eight patients with PNH, the majority of monocytes did not express the CD14 antigen (76% to 100% of negative cells). In eight of 13 patients with AA in whom a GPI defect was detected on granulocytes, a small proportion of CD14-monoctyes (9% to 44%) was also detected. In two AA patients with markedly defective CD16 and CD67 expression (see above), most monocytes were also CD14- (95% and 99%). Two patients had peripheral monocytopenia that precluded meaningful flow-cytometry analysis. The percentage of affected monocytes was similar to that of affected granulocytes, except in the case of one patient (no. 48) with PNH, in whom the proportion of GPI-deficient monocytes was higher than the proportion of affected granulocytes (Fig 2 and Table 2).

Analysis of Lymphocytes

Lymphocytes were tested with the CD48 GPI-linked molecule. Lymphocytes were recognized due to their light-scatter pattern and by CD2/CD4 double-staining. Three of eight patients with PNH were tested for CD48 expression, and two (no. 50 and 47) were deficient in CD48 expression (70% and 84% of negative cells, respectively) as compared with one of 12 AA patients who secondarily developed PNH and none of 25 AA patients without a PNH clone.

Follow-Up Study

In the first part of this study, the GPI defect on red blood cells was only indirectly assessed by the Ham and sucrose tests. This made it difficult to compare the proportion of erythrocytes affected with the number of granulocytes affected. We thus initiated a follow-up study of our patient cohort, including the above-studied GPI-anchored molecules on granulocytes and monocytes, and added the study of CD55, CD58, and CD59 on erythrocytes. We first confirmed that CD55, CD58, and CD59 molecules were indeed expressed on almost 100% of erythrocytes (10 blood donors and one patient with congenital AA). Two patients with PNH and 14 with AA were studied for 6 to 13 months (mean, 12) in the follow-up study.

AA patients: Among 14 patients with AA, four (no. 3, 14, 16, and 35) had no GPI-anchoring defect in the first study and remained free of a defect in the follow-up study in all blood-cell subsets (including erythrocytes). In one of these four patients (no. 3), Ham or sucrose tests were initially weakly positive and dissociated. He remained free of a GPI-anchoring defect on both leukocytes and erythrocytes, and 10 months after the initial study, had negative Ham and sucrose tests.

In seven patients, a GPI defect on monocytes and granulocytes had been detected previously. In the follow-up study, the proportion of defective granulocytes and monocytes re-
mained roughly the same in six patients. A GPI-anchoring
defect was detected on erythrocytes in five patients with
positive Ham and sucrose tests. In one patient (no. 1), who
had a GPI-anchoring defect on leukocytes, no defect was
observed on erythrocytes (Ham and sucrose tests were also
negative) (Table 3). The seventh patient (no. 40) underwent
allogeneic BMT. Seven and 9 months after transplant, flow-
cytometry analysis demonstrated disappearance of the PNH
clon.
In three patients (no. 22, 26, and 49) without a GPI-an-
choring defect after the initial study, a defect was detected
in the follow-up study. This defect was limited to leukocytes
with persistent negative Ham and sucrose tests in patient no.
22. It was found in all cell subsets with Ham and sucrose
tests, becoming positive in patient no. 26, and on erythro-
cytes only (isolated reduced expression of the CD59 mole-
cule), with persistent negative Ham and sucrose tests in pa-
tient no. 49.

PNH patients. For the two patients with PNH, the GPI-
defect on leukocytes was of the same order of magnitude as
in the initial study. The defect on erythrocytes reached 35%
and 75%, on average, for patients no. 44 and 51, respectively.

Ham and Sucrose Tests: Correlation With Flow-Cytometry
Analysis

Ham and sucrose tests and flow-cytometry analysis were
performed on the same day in the absence of any recent
blood-product transfusion. In all but five patients, a good
correlation was observed between the positivity of Ham and
sucrose tests and a GPI-linked molecule defect on granulo-
cytes and monocytes (Table 2). In two patients with AA (no.
3 and 13) in whom the Ham or sucrose test was weakly
positive and dissociated (Table 2), no PNH defect could be
detected on granulocytes and monocytes by immunofluores-
cence. One of these patients (no. 3) was recently reevaluated
as part of the follow-up study (see previous section). In
contrast, defective GPI molecule expression was found on
leukocytes in three patients with AA (no. 1, 24, and 40)
whose Ham and sucrose tests were negative. One patient
(no. 1) was reevaluated in the follow-up study. Twelve
months after the first study, we confirmed a GPI-anchoring
defect on granulocytes and monocytes, while erythrocytes
were not affected (see previous section).

Cytogenetic Studies

Cytogenetic analyses were performed in 15 cases (12 pa-
tients with AA and three with PNH). Monosomy 7 was
diagnosed in an AA patient who had no GPI defect. One
patient with PNH had a constitutional abnormality [Robert-
sonian translocation t(13;14)].

Coagulation Tests

Proteins C and S, thrombomodulin, and antithrombin III
levels were within the normal range in the group of patients
with PNH and in patients with AA (with or without PNH) (Table 4).

Study of PIG-A Gene Expression by Northern Blot Analysis

Sufficient RNA could be recovered from frozen PBMC in only five of eight patients with PNH. In four of these patients, the PIG-A transcript was barely visible in two and undetectable in two (Table 2 and Figs 3 and 4). An abnormally small transcript was detected in the remaining patient (no. 47). The PIG-A transcript was analyzed in nine of 12 patients with AA in whom a GPI defect was detected by flow cytometry. A normal-sized transcript was observed in all nine (Table 3). The level of PIG-A gene expression was quantified by the PIG-A/β-actin gene ratio using densitometry, and was apparently reduced, since in both healthy blood donors and AA patients, the ratio was 1.93 arbitrary units (range, 1.10 to 2.42) and was 0.46 arbitrary units (range, 0.25 to 0.8) in eight patients with AA in whom a GPI defect was detected by flow cytometry.

DISCUSSION

AA and PNH are clinically related syndromes. During the course of PNH, pancytopenia is common and about one third of patients will die of bone marrow failure. Similarly, a fairly high proportion of patients with pancytopenia and marrow hypocellularity have either a positive sucrose hemolysis test or Ham test during the evolution of the disease. The overlap with AA is particularly striking among younger patients with PNH, who much less frequently develop hemoglobinuria and more often have moderate to severe marrow failure, often leading to an initial diagnosis of AA. In addition to their concurrent or sequential appearance in individual patients, PNH and AA share several pathophysiologic features. Hematopoietic progenitor numbers are markedly decreased in patients with AA and PNH, even when the marrow appears cellular. However, the question as to whether these two disorders are two forms of the same disease is unresolved. To search for a pathogenetic link between these two syndromes, we compared patients with PNH with those with AA who secondarily developed PNH after IST, using the results of Ham and sucrose tests at diagnosis as the classic diagnostic criteria.

All patients with PNH had a mixture of the classic clinical manifestations (hemolytic anemia, thrombosis, and AA), while only six of 12 AA patients who developed PNH had clinical symptoms (essentially hemolytic anemia). The other six patients had a purely laboratory-based diagnosis. The Basel group has also reported that four of their 13 patients who developed PNH after IST were diagnosed by laboratory tests while they were free of clinical manifestations.

During the past few years, flow-cytometry analysis has proved to be a powerful tool for the diagnosis of the GPI-linked protein defect in PNH. As in this report, the various cell lineages studied include granulocytes (using CD16/CD67), monocytes (using CD14), erythrocytes (using CD55/CD59), and lymphocytes (using CD48). Although the nature of the primary disease (PNH or AA) is generally omitted from such reports, it seems that patients with PNH exhibit a pronounced GPI-linked protein defect. We found that 87.5% of monocytes and 84% of granulocytes had a GPI defect in patients with PNH. In contrast, in 10 of 12 AA patients who secondarily developed PNH, the defect was less pronounced, affecting a mean of 27% and 20% of monocytes and granulocytes, respectively. In the remaining two patients, the GPI-linked protein defect reached the same level as that found in PNH patients (>90%). Such a quantitative difference in the extent of the defect in these two forms of the disease has also been noted by others. The involvement of lymphocytes in the GPI-anchoring defect has been the subject of conflicting reports with lymphocytes being affected in 11 of 12 patients in the study by Schubert et al., compared with only two of 10 patients in the report by Van der Schoot et al. In our study, two of three patients with PNH and one of 12 patients who secondarily developed PNH exhibited a CD48 defect on lymphocytes. Further studies are clearly warranted to determine whether both lymphocytes and myeloid involvement (thus suggesting a stem-cell level defect) are more frequent in PNH.

Table 3. Flow-Cytometry Analysis: Summary of the Follow-Up Study

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Ham Test</th>
<th>Sucrose Test</th>
<th>% of Negative Cells</th>
<th>Interval First Study/Follow-Up Study (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AA</td>
<td>-/--</td>
<td>16/6-6/0-0-0</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AA</td>
<td>-/+</td>
<td>16/5-5/0-0-34*</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AA</td>
<td>+/+</td>
<td>40/39-38/21-48-47</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AA</td>
<td>(+)(+)(+)</td>
<td>35/51-65/11-37</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AA</td>
<td>++/+</td>
<td>93/92-93/33-58-88</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>AA</td>
<td>+/-</td>
<td>NA/33-32/22-18-80</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>AA</td>
<td>-/-</td>
<td>NA/37/30/0-0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>AA</td>
<td>(+)/(+)</td>
<td>35/12-12/12-16-49</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>AA</td>
<td>-/-</td>
<td>0/0/0-0-0-33*</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>PNH</td>
<td>+/-</td>
<td>90/95-94/34-35-34</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>PNH</td>
<td>+/-</td>
<td>90/88-88/75-73-68</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Results of lytic tests are given in pluses; corresponding values in the percentage of lysis are described in Materials and Methods.

* Protein reduced, rather than absent on abnormal cells.

Table 4. Coagulation Test Results

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Protein C Activity (%)</th>
<th>Protein C Antigen (%)</th>
<th>Protein S Total (%)</th>
<th>Protein S Free Form (%)</th>
<th>Antithrombin III (%)</th>
<th>Thrombomodulin (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNH</td>
<td>119.1 ± 43.0</td>
<td>99.3 ± 20.6</td>
<td>84.7 ± 14.2</td>
<td>92.9 ± 17</td>
<td>106 ± 13.8</td>
<td>28.3 ± 11.7</td>
</tr>
<tr>
<td>AA</td>
<td>88.5 ± 14.2</td>
<td>79.1 ± 6.8</td>
<td>78.6 ± 5.7</td>
<td>83.8 ± 7.6</td>
<td>NA</td>
<td>46.8 ± 26</td>
</tr>
<tr>
<td>Controls</td>
<td>82.6 ± 17.2</td>
<td>83.4 ± 13.5</td>
<td>88.9 ± 10.8</td>
<td>89.9 ± 22.8</td>
<td>87.2 ± 18.2</td>
<td>39.8 ± 24.5</td>
</tr>
</tbody>
</table>
In the follow-up study of our patient cohort, we added the study of CD55, CD58, and CD59 on erythrocytes to that of GPI-anchored molecules on granulocytes and monocytes. To date, two patients with PNH and 14 with AA have been studied for 6 to 13 months in the follow-up study. Among patients with AA, four in whom no GPI-anchoring defect was detected in the first study had no defect at follow-up study in any of the blood-cell subsets (including erythrocytes). Analysis of granulocytes, monocytes, and erythrocytes was performed in seven of 13 AA patients in whom affected monocytes and granulocytes were previously detected. In the follow-up study, the proportion of defective granulocytes and monocytes remained roughly the same in six patients (the remaining patient underwent allogeneic BMT). A GPI-anchoring defect was detected on erythrocytes in five patients with positive Ham and sucrose tests. However, a defect was not found on erythrocytes in one patient (no. 1) who had a GPI-anchoring defect on leukocytes. The involvement of granulocytes and monocytes without involvement of erythrocytes has been reported in one patient and granulocytes were also the first cells affected in 11 of 29 AA patients with a GPI-anchoring defect recently reported by Schubert et al. In three patients without a GPI-anchoring defect after the initial study, a defect was subsequently detected on leukocytes only in one patient, in all cell subsets in another patient, and on erythrocytes only in the last one. We found some discrepancies in the percentages of negative red blood cells with different antibodies in this last case, as well as in some other AA cases (see Table 3).

Few studies have used more than one antibody to study a GPI defect on red blood cells in a large number of AA patients. Such discrepancies, including a more marked deficiency in CD59 antigen, have also been noted by others. This might be due to differences between CD55, CD58, and CD59 in the structure of the anchorage site, as suggested by others. Furthermore, this follow-up study illustrates that there is no evidence of the abnormal clone early in the development of the disease in some patients with AA who ultimately had PNH and no evidence of such a clone in the remaining patients.

A good correlation was found between the results of Ham and sucrose tests and those of flow cytometry in all but five patients in the initial study. The Ham and sucrose tests were negative in three patients who, on the same day, exhibited a GPI defect on granulocytes and monocytes. In the follow-up study, one of these three patients was reevaluated. We confirmed a GPI-anchoring defect on granulocytes and monocytes, while erythrocytes were not affected, as reported in two other cases. On the other hand, no GPI-defect was found in two patients who had dissociated positivity for Ham and sucrose tests. One of these patients was recently reevaluated as part of the follow-up study. He remained free of a GPI-anchoring defect on both leukocytes and erythrocytes, 10 months after the initial study, and had negative Ham and sucrose tests.

Cytogenic features were normal in all but one PNH patient [constitutional abnormality; der(13;14)]. One patient with AA, but without PNH, had monosomy 7 associated with a myelodysplastic syndrome, another well-recognized complication that occurs after IST.
The pathophysiologic factors involved in the occurrence of thrombosis in patients with PNH are not yet fully elucidated. A recent study showed that the lack of the receptor for urokinase-type plasminogen activator observed on PNH leukocytes may be related to the increased tendency toward thrombosis. A protein C/protein S/thrombomodulin system impairment and antithrombin III deficiency are considered the main causes of thrombophilia. To test this hypothesis, we determined protein S, protein C, antithrombin III, and thrombomodulin plasma levels in patients with PNH and AA. Proteins C and S, thrombomodulin, and antithrombin III levels were within the normal range in PNH patients and in those with AA (with or without PNH). Another hypothesis for the increased risk of thrombosis in PNH patients is through platelet activation. In this regard, it is interesting to note that it was recently demonstrated that PNH platelets with undetectable levels of CD59 antigen show increased sensitivity to activation by C5b-9 proteins compared with normal controls, resulting in markedly increased production of procoagulant vesicles. This may contribute, at least in part, to the tendency toward thrombosis in PNH patients.

PIG-A gene expression studied by Northern blot analysis showed that among five patients with PNH, from whom sufficient RNA could be recovered from PBMC for analysis, the PIG-A transcript was barely visible in two, undetectable in two, and abnormal in length in the fifth. In these five patients, a GPI-anchoring defect affected the majority of monocytes. Lymphocytes were studied in two of these five patients, and in these two patients, 70% and 84% of the lymphocytes had a GPI-anchoring defect. Thus, the mRNA extracted from PBMC originated mostly from affected cells (at least in the two patients in whom both monocyte and lymphocyte populations could be studied). The fact that the PIG-A transcript was barely visible in two cases and undetectable in two further cases may reflect to a considerable extent, instability of the affected transcripts. Although Ware et al found a normal level of PIG-A transcript in four patients, Takeda et al found barely detectable PIG-A gene expression in GPI-deficient lymphoblastoid cell lines. Our fifth patient (no. 47) had an abnormally small transcript, similar to that described in a patient by Takeda et al. Recently, Bessler et al described three different PIG-A mRNA species in four cell lines deficient in GPI-linked proteins. The two smaller mRNA species (2.8 and 3.3 kb), which were not detected in our analyses, are probably present in a small amount and could probably only be detected in the poly(A)-enriched RNA fraction. The exact molecular alterations (ie, point mutations or deletions) responsible for the reduction in the amount of full-length mRNA or for its abnormal length remain to be determined. Using reverse-transcribed amplification of the PIG-A transcripts and subsequent cloning in plasmids, the Osaka group further extended their sequencing studies in granulocytes isolated from five patients with PNH and, recently, Ware et al also demonstrated point mutations within the coding regions of the PIG-A gene in four patients with PNH.

In conclusion, the only significant difference we were able to establish in this study, between PNH arising in patients with previous AA and patients with de novo PNH, was the size of the PNH clone. Whether the PNH clone already exists in some AA patients at diagnosis but is undetectable by the methods used in this study remains to be determined by more sensitive techniques. Whether a PIG-A gene alteration is also the underlying genetic defect in otherwise typical AA also seems to warrant attention.
ACKNOWLEDGMENT

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Aplastic anemia and paroxysmal nocturnal hemoglobinuria: search for a pathogenetic link

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