Deficiency of Glycosyl-Phosphatidylinositol-Linked Membrane Glycoproteins of Leukocytes in Paroxysmal Nocturnal Hemoglobinuria, Description of a New Diagnostic Cytofluorometric Assay

By C. Ellen van der Schoot, Tom W.J. Huizinga, Elisabeth T. van’t Veer-Korthof, Rosemarie Wijmans, Jantina Pinkster, and Albert E.G. Kr. von dem Borne

Paroxysmal nocturnal hemoglobinuria (PNH) is a disease that affects not only red cells, but other blood cells as well. The common defect is supposed to be an acquired deficiency of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins, which may be present already at the hematopoietic stem cell level. Recently, a panel of monoclonal antibodies (MoAbs) has become available directed against various GPI-linked membrane proteins. This makes it possible to study various cell lineages for the deficiency of such proteins in PNH in more detail. Using cytofluorography, we could show that the granulocytes of 20 different PNH patients miss not only GPI-linked FcRIII (CD16 antigen), but also three other GPI-linked proteins, ie, CD24 antigen, CD67 antigen and a granulocyte-specific 50 to 80 Kd antigen. The affected granulocytes were not only neutrophils but also eosinophils, as was found in a more detailed analysis of three patients. Moreover, in all 10 PNH patients tested, the monocytes were found to be deficient for the GPI-linked CD14 antigen, and we found with CD24 and CD55 (DAF) antibodies that lymphocytes may be involved as well. However, abnormal B and T lymphocytes were detected only in a subset of patients (2 of 10 tested). The uniform deficiency of GPI-linked proteins of granulocytes allows the introduction of a new diagnostic cytofluorometric assay for PNH with MoAbs against GPI-linked granulocytic antigens. This test was positive in all PNH patients studied and not in a group of 40 control patients or 50 normal donors, with the exception of three of 16 aplastic anemia (AA) patients. In the three AA patients, subpopulations (10% to 20%) of PNH granulocytes could be detected, whereas these patients had a negative acidified serum (Ham) test. This indicates that the new test is more sensitive than the Ham test and allows the early diagnosis of PNH in AA. An advantage of the neutrophil assay is that, in contrast to the Ham test, it is not influenced by recent red-cell transfusions. Moreover, it is possible to quantify the number of affected cells by single cell analysis.

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antigen), B lymphocytes (CD24 antigen and DAF), T lymphocytes (DAF), and monocytes (CD14 antigen), we found that the membrane lesion specific for PNH could be demonstrated on these cells as well. Because neutrophils, eosinophils, and monocytes deficient for GPI-linked proteins could be demonstrated in all PNH patients tested, but only in some patients affected lymphocytes, we assume that in PNH the abnormal cells are always derived at least from commonly affected progenitors (CFU-GEMM). Therefore, it is possible to introduce a new diagnostic test for PNH based on the cytofluorographic analysis of the reactivity of MoAbs against GPI-linked proteins on leukocytes and specifically on the easily testable granulocytes. In contrast to the standard diagnostic red cell assays for PNH, the reliability of this assay will not be influenced by previous red cell transfusions, which are often administered to PNH patients. Moreover, it is possible to quantify the percentage of affected cells by single cell analysis.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained from 12 patients with previously diagnosed PNH and 8 patients with newly diagnosed PNH. The latter 8 patients came from a series of 48 patients with clinical suspicion for PNH, whose blood was sent to our laboratory for routine screening in the acidified serum (Ham) test. In 17 of these patients, the diagnosis was confirmed by a positive Ham test, whereas in 2 patients the Ham test (as well as the sucrose lysis test) were only dubiously positive. In one patient, the Ham test and the sucrose lysis test were negative. However, in this patient the acetylcholinesterase content of the erythrocytes was lowered (0.23 μmol DTNB/min/L, normal values 0.25 to 0.40 μmol). Blood from 50 normal donors was analyzed as well. Furthermore, peripheral blood was obtained from 16 patients (all children with aplastic anemia with no clinical signs of increased hemolysis. None of these patients had a positive Ham test, but in two patients the sucrose lysis test was positive. The cells of two patients with the rare congenital disorder hereditary erythrocytic multinucularity with positive acetylcholinesterase activity were included in this study. They were used as controls, where the presence of GPI-linked antigens could be demonstrated in all PNH patients tested, but only in some patients affected lymphocytes, we assume that in PNH the abnormal cells are always derived at least from commonly affected progenitors (CFU-GEMM). Therefore, it is possible to introduce a new diagnostic test for PNH based on the cytofluorographic analysis of the reactivity of MoAbs against GPI-linked proteins on leukocytes and specifically on the easily testable granulocytes. In contrast to the standard diagnostic red cell assays for PNH, the reliability of this assay will not be influenced by previous red cell transfusions, which are often administered to PNH patients. Moreover, it is possible to quantify the percentage of affected cells by single cell analysis.

Cell separation. Cells were isolated from heparin-anticoagulated peripheral blood. By Ficoll-Isopaque (δ = 1.077 g/cm³) centrifugation the mononuclear cells were separated from the granulocytes. The red cells were removed by lysis in NH₄Cl. The granulocytes were tested directly, the mononuclear cells were tested either directly or after cryopreservation in a medium containing 10% DMSO and 20% fetal calf serum (FCS). In the mononuclear cell fraction, monocytes were recognized by their forward and side scatter in the FACSCAN, T lymphocytes by CD3 antigen, B lymphocytes by CD20 antigen, NK cells by CD56 antigen, and eosinophils by CD9-antigen expression, respectively.

Immunofluorescence. The cells were fixed in 1% (wt/vol) paraformaldehyde except for erythrocytes, which were tested unfixed. They were then incubated with the MoAbs in appropriate dilutions, washed with phosphate buffered saline (PBS) containing 0.2% (wt/vol) bovine serum albumin (BSA) and subsequently incubated with FITC-conjugated goat-anti-mouse Ig (GM17-01 FF, CLB) or phycoerythrin (PE)-conjugated rat-anti-mouse kappa-chain (purchased from Becton Dickinson, Mountain View, CA). In case of double fluorescence experiments, the free antigen-binding sites of the second antibody were blocked with irrelevant murine IgG1 and IgG2a MoAbs. Thereafter, the cells were incubated with a directly conjugated antibody. The fluorescence intensity (FI) was quantified by flow cytometry (FACSCAN). When cells were studied in double-fluorescence procedures, at least 2,000 cells that were reactive with the MoAb applied to recognize the leukocyte subset were analyzed. The mean fluorescence intensity for the different antibodies was expressed in arbitrary units on a linear scale after subtraction of the mean fluorescence of cells incubated with an irrelevant isotype-matched antibody. The following MoAbs were used: Leu4-PE (CD3, IgG1), CLBThromb/8 (CD9, IgG2a), CLB-mon/1 (CD14, IgG2a), CLB-B4.3 (CD15, IgM), CLBFCRgran/1 (CD16-FcRIII, IgG2a), Leu11-PE (CD16-FcRIII, IgG1), Leu16-PE (CD20, IgG1), CLBgran/Bly/1 (CD24, IgG1), ES11CV (CD36, IgG1), BRIC110 (CD55-DAF, IgG1), Leu19-PE (CD56, IgG1), BRIC5 (CD58-LFA-3, IgG), YTH53.1 (CD59-MIRL, IgG1), B13.9 (CD67, IgG1), CLBgran/10 (CD66, IgG1), and the antibody CLBgran/5 (unclustered, IgG1), directed against a 50 to 80-Kd GPI-linked antigen of neutrophils. The BRIC antibodies were kindly donated by Dr D. Anstee (Oxford, UK), YTH53.1 was a gift of Dr H. Waldmann (Cambridge, UK). Leu4-PE, Leu11-PE, Leu16-PE, and Leu19-PE were purchased from Becton Dickinson. The other antibodies were produced in our own laboratory and, except for CLBgran/5, clustered in the International Workshops on Leucocyte Differentiation Antigens. The monoclonal antibodies against PI-linked antigens are listed in Table 1.

RESULTS

GPI-linked glycoprotein deficiencies of PNH granulocytes (neutrophils, eosinophils). The Ficoll-Isopaque granulocyte fraction from the blood of all patients was tested by cytofluorography for the expression of the GPI-linked CD16 (FcRIII), CD24, CD67, and CLBgran/5 antigens. This fraction was not tested routinely with CD55 (anti-DAF) or CD58 (anti-LFA-3) antibodies. DAF and LFA-3 are also GPI-linked membrane proteins. However, their expression on granulocytes is too low for reliable cytofluorographic analysis (mean FI, 20 to 40, compared with a mean FI of 200 to 500 for the other antigens). To make sure that the analyzed cells (gated on differences in light scattering) were indeed granulocytes, they were also tested for the expression of the granulocyte-specific non-GPI-linked CD15 and CD66 antigens. The granulocyte fraction consists mainly of neutrophils and contains only a small number of eosinophils (1.5% to 5%). Thus, results of the overall cytofluorographic analysis of this fraction reflect mainly but not entirely the neutrophil antigen make-up. FcRIII (CD16 antigen) and CLBgran/5 antigens are expressed only on neutrophils and not on eosinophils. Therefore, also in normal donors a small population of granulocytes negative for these antigens exists.

Table 1. PI-Linked Proteins of Blood Cells

<table>
<thead>
<tr>
<th>CD</th>
<th>Antigen</th>
<th>MoAb</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>55 Kd</td>
<td>CLBmon/1</td>
<td>Monocytes</td>
</tr>
<tr>
<td>16</td>
<td>FcRIII</td>
<td>CLBFCRgran/1</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>24</td>
<td>38/41 Kd</td>
<td>CLBgran/Bly/1</td>
<td>Neutrophils, eosinophils, B lymphocytes</td>
</tr>
<tr>
<td>55</td>
<td>DAF</td>
<td>BRIC110</td>
<td>Leukocytes, eosinophils, B lymphocytes</td>
</tr>
<tr>
<td>58</td>
<td>LFA-3</td>
<td>BRIC5</td>
<td>Leukocytes, eosinophils, B lymphocytes</td>
</tr>
<tr>
<td>59</td>
<td>MIRL</td>
<td>YTH53.1</td>
<td>Leukocytes, eosinophils, B lymphocytes</td>
</tr>
<tr>
<td>67</td>
<td>100 Kd</td>
<td>CLBgran/5</td>
<td>Neutrophils, eosinophils, B lymphocytes</td>
</tr>
<tr>
<td>69</td>
<td>50–80 Kd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In all 20 PNH patients granulocytes were found that were deficient for all the GPI-linked antigens. Besides this subset of affected granulocytes (PNH-G), in all but two PNH patients granulocytes with normal expression of GPI-linked antigens were present. The percentage of PNH-G in the different patients varied from 30% to 100% (Fig 1). These percentages were similar for the different antibodies. The reactivity of the antibodies with the PNH-G as measured by fluorescence intensity, is shown in Fig 2. As expected, the expression of the CD15 and CD66 antigens was normal. Not in all patients the PNH-G were completely deficient for the GPI-linked CD24, CD67, and CLBgran/5 antigens. Although the measured fluorescence was extremely low in all cases, the PNH-G showed more than background reactivity (arbitrarily defined as FI > 8) in these patients, notably with CD24. In all 20 patients, the PNH-G still expressed FcRIII (CD16) (FI from 15 to 80). Fluorescence intensity induced by CD16 on the PNH-G showed a log normal distribution. This means that only one subpopulation of similarly deficient granulocytes existed in every patient tested.

The eosinophils of three PNH patients were studied in detail. Eosinophils were recognized by the expression of CD9 antigen and their characteristic light scatter. CD9 antigen was shown to be expressed on eosinophils and not on neutrophils. In all three patients, subpopulations of CD67 and CD24 antigen-deficient CD9-positive eosinophils were
found. An example of the characteristic fluorescence pattern of the different antibodies on the different cell types in PNH is shown in Fig 3.

GPI-linked glycoprotein deficiencies of PNH mononuclear cells (monocytes, B and T lymphocytes). Besides granulocytes, we tested the monocytes, and B and T lymphocytes of 10 PNH patients and 20 patients of the control group. Monocytes were recognized by their characteristic light-scatter pattern and by the expression of CD36. In all 10 patients, we found monocytes that did not express the GPI-linked CD14 antigen. However, for unknown reasons, always only small numbers of monocytes were present in the mononuclear cell fraction of PNH patients. Moreover, the CD14 antigen is expressed on only 80% to 90% of the monocytes of normal donors. Thus, it was impossible to determine the actual percentage of affected monocytes in our PNH patients. B and T lymphocytes were tested in double-color fluorescence with Leu16 (CD20) and Leu4 (CD3), respectively, for the expression of the GPI-linked CD24 antigen and DAF. As shown in Table 2, in two patients we found subpopulations of CD24- and DAF-negative B cells (25% and 15%) and DAF-negative T cells (15% and 15%). In all other patients, as well as in the control group, no subpopulations of B and T lymphocytes deficient in GPI-linked proteins were detected. We were not able to study NK cells, because no suitable antibodies were available for this purpose. The expression of DAF on NK lymphocytes of normal donors is extremely low and in some individuals even undetectable. The FcRIII on NK lymphocytes is not GPI-linked. Neither were suitable reagents available to study GPI-linked proteins of basophils from our PNH patients.

GPI-linked glycoprotein deficiencies of PNH-erythrocytes. To compare the percentage of PNH-G with the percentage of PNH erythrocytes (PNH-E) in six PNH patients, erythrocytes were tested with MoAbs against DAF (CD55), LFA-3 (CD58), and MIRL (CD59) by immunofluorescence. The expression of DAF was decreased on the erythrocytes of all PNH patients. However, due to the low expression of DAF on normal erythrocytes no clear discrimination between normal and PNH-E could be made. With the more strongly reactive anti-MIRL and anti-LFA-3 antibodies in all PNH patients subpopulations of at least 15% deficient erythrocytes were found (Table 2). In 30 normal donors greater than 98% of the erythrocytes expressed normal amounts of these GPI-linked proteins. In all cases studied the percentage of affected red cells was lower than the percentage of PNH-G. More than one abnormal subpopulation of red cells was not found in these patients.

Sensitivity and specificity of cytofluorometric assay on granulocytes. We also studied the specificity and sensitivity of immunofluorescence cytofluorometry for the diagnosis of PNH. The MoAbs against the different GPI-linked proteins of granulocytes were used to analyze not only the cells of the 20 PNH patients but also of 40 control patients (patients with clinical suspicion for PNH, but with a negative Ham test) and of 50 normal donors. As can be seen in Fig 1, in the control patients or in the healthy donors populations of

### Table 2. Percentages of Affected Cells in Six PNH and Three Aplastic Anemia Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Granulocytes (%)</th>
<th>Erythrocytes (%)</th>
<th>B Lymphocytes (%)</th>
<th>T Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNH-1</td>
<td>95</td>
<td>65</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PNH-2</td>
<td>100</td>
<td>51</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>PNH-3</td>
<td>85</td>
<td>49</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PNH-4</td>
<td>83</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PNH-5</td>
<td>75</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PNH-6</td>
<td>56</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AA-1</td>
<td>19</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AA-2</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AA-3</td>
<td>12</td>
<td>NT</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.
neutrophils deficient in GPI-linked proteins were not found. Thus, the cytofluorometric test is both sensitive and specific in the diagnosis of PNH. In a group of 16 patients (children) with aplastic anemia, small subpopulations of affected neutrophils were detected in three patients (10%, 12%, and 19%, respectively). The Ham test was negative in all these three cases, the sucrose lysis test was positive in two of the three. In two of these patients (including the patient with a negative sucrose lysis test) the erythrocytes were tested in the immunofluorescence test. In both patients PNH-E could be detected (Table 2). These children did not have clinical signs of increased hemolysis and were not transfusion-dependent. These patients will be described in more detail elsewhere. Two HEMPAS patients were also studied and showed normal expression of GPI-linked proteins on their neutrophils (data not shown).

**DISCUSSION**

In the present study we show that, in addition to the previously described deficient GPI-linked membrane structures alkaline phosphatase,17 LFA-3 (CD58),16 DAF (CD55),8 and FcRIII (CD16),22,23 the GPI-linked CD24, CD67 antigens and a 50 to 80-Kd protein are deficient on PNH granulocytes. So far, all granulocyte antigens that have been identified as GPI-anchored proteins are deficient in PNH. These data strongly support the hypothesis that the common defect in PNH is the deficiency of all GPI-anchored membrane proteins.

The FcRIII (CD16) was not totally absent from the granulocytes (mean FI 15 to 80 U, Fig 2), whereas the other three antigens were, in most cases, completely absent. In the blood, FcRIII is expressed on neutrophils and NK cells. The FcRIII of NK cells is a transmembranous form (FcRIII-2). In contrast, the neutrophils have a GPI-linked form of FcRIII (FcRIII-1).28 Therefore, the expression of the FcRIII on PNH neutrophils is unexpected. Compared with the other GPI-linked antigens, the fluorescence intensity obtained with MoAb CLBfcrgran/1 (CD16) on normal neutrophils is high due to the presence of 100,000 to 300,000 FcRIIs per neutrophil.25 Thus, a possible explanation is that the defect in PNH is never complete and that the remaining expression is always above the detection limit only for FcRIII. However, we were not able to reduce the expression of CD16 antigen by digestion of the PNH neutrophils with glycosylphosphatidylinositol-specific phospholipase (GPI-PLC) (results not shown). Another explanation is that on neutrophils, besides the GPI-linked forms, other forms exists that are bound to or in the membrane in yet an unknown way. Recently, such alternate binding for FcRIII was shown by Kuroskii et al in an experimental system.30 The FcRIII-1 precursor could associate with the γ-chain of FcRβ and become expressed in this way on the surface of COS cells.

It has been hypothesized that the differences in complement sensitivity in PNH type-II and type-III red cells are due to qualitative differences between these cell types. Type-III cells would have additional deficiencies for proteins other than DAF (CD55 antigen), such as C8bp and MIRL (CD59 antigen). However, recently, it has been shown that PNH-E type II are deficient for MIRL as well,31 which suggests that only subtle quantitative differences in membrane complement regulatory proteins underlie the variability in complement sensitivity of PNH erythrocytes. Our results on granulocytes and erythrocytes are in agreement with this latter explanation. We did not find subsets of differently affected granulocytes or erythrocytes in any patient, whereas we did find relatively subtle quantitative differences in the expression of GPI-linked membrane glycoproteins between the different patients (notably on granulocytes). Possibly in some patients the deficiency is not complete and leading to some expression of GPI-linked proteins.

The existence of abnormal granulocytes in PNH have previously been described.14,17 In agreement with this observations we found that in all our 20 patients deficient neutrophils were present in the blood, though we cannot exclude that in rare patients the neutrophils are normal. Furthermore, we demonstrate for the first time that of the granulocytes not only the neutrophils but also the eosinophils are affected in PNH. This finding further supports the stem-cell origin of this disease, as does the fact that monocytes of all 10 patients tested were also affected, as demonstrated by the deficiency of CD14 antigen.

Originally, it was thought that lymphocytes do not have the membrane lesions characteristic of PNH.2,5,12 However, Kinoshita et al4 found that lymphocytes from two of three PNH patients showed decreased expression of DAF. We found in two of 10 patients low percentages (15% to 25%) of T lymphocytes that were deficient for DAF (CD55 antigen). Furthermore, we demonstrated that B lymphocytes may be involved in PNH as well. In the same two patients with abnormal T lymphocytes, we found also B lymphocytes deficient for the CD24 and DAF antigen.

These findings indicate that, in at least these two patients, the disease arises in the totipotent stem cell. It was striking that both patients with abnormal lymphocytes were patients of whom 100% of the neutrophils were abnormal (Table 2). The low percentages of abnormal lymphocytes may explain the negative results in previous studies.

The standard diagnostic assays for PNH are based on the abnormal sensitivity of the red cells for spontaneously activating complement, such as in acidified serum31 or to complement activated by the classic pathway.34 The diagnosis can be confirmed by the detection of a decreased acetylcholine-esterase content of the erythrocytes.15 All these assays are based on abnormalities of the red cells. Therefore, the reliability of these tests is influenced by previous red-cell transfusions. Moreover, a decrease in acetylcholine-esterase activity has been found in other diseases as well.35 The cytofluorographic analysis of granulocytes might circumvent these problems. An additional advantage is that the percentage of affected cells can be quantified, because the differences in expression of the GPI-linked antigens that we studied between normal and abnormal cells were quite prominent. To test the specificity of the immunofluorescence assay, we tested the granulocytes of 60 patients with PNH, or clinical suspicion for PNH, in parallel with the Ham test on their erythrocytes. In only one patient did we find a discrepancy, eg, a negative Ham test result and PNH-G in the immunofluorescence assay. However, in this patient, who
had 50% abnormal circulating granulocytes, the diagnosis PNH could be confirmed by showing that the erythrocytes had a decreased acetylcholine-esterase content. Furthermore, discrepancies were found in the group of 16 patients with aplastic anemia (AA). In three of the 16 AA patients, we found a subpopulation of PNH-G (10% to 20%), whereas the Ham test was negative in all. Lewis and Dacie originally described the association of PNH and AA. Approximately 15% of the patients with AA develop PNH in the course of their disease. Therefore, we assume that our three patients may develop clinical PNH in due time. The discrepancy between the two tests possibly reflects the higher sensitivity of the cytofluorographic analysis.

We also studied two patients with a positive Ham test due to hereditary erythrocytic abnormalities (HEMPAS; data not shown). Both had only normal granulocytes in the immunofluorescence assay. Thus, the neutrophil GPI-linked membrane protein test also allows an easy discrimination between two different types of patients with positive Ham tests, ie, PNH and HEMPAS.

In conclusion, in PNH neutrophils, eosinophils, and monocytes are deficient for all presently known GPI-linked membrane proteins, as well as in some patients B and T lymphocytes. Based on these findings, we developed a simple cytofluorometric assay on granulocytes which appeared to be diagnostic for PNH and also made the diagnosis of PNH in aplastic anemia possible.

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