Discordant and Heterogeneous Expression of GPI-Anchored Membrane Proteins on Leukemic Cells in a Patient With Paroxysmal Nocturnal Hemoglobinuria

By Tsutomu Shichishima, Takashi Terasawa, Chokichi Hashimoto, Hitoshi Ohto, Masuhiro Takahashi, Akira Shibata, and Yukio Maruyama

We performed a flow cytometric analysis using monoclonal antibodies to decay accelerating factor (DAF) and CD59/membrane attack complex inhibitory factor (CD59/MACIF) in order to investigate the leukemic cells and erythrocytes from a patient with paroxysmal nocturnal hemoglobinuria (PNH) who developed acute myelocytic leukemia. In May 1990, the leukemic cells comprised 70% of the mononuclear cells in the bone marrow and 76% of those in the peripheral blood. They consisted of a mixture of positive and negative populations, including single DAF-positive cells. In August 1990, almost 100% of the peripheral mononuclear cells were leukemic blasts, and these consisted of a single population with reduced DAF expression. Single-color flow cytometric analysis showed that the leukemic cells lacked CD59/MACIF, while control leukemic cells (n = 3) expressed both DAF and CD59/MACIF. Leukemic blasts from this patient and six control patients expressed lymphocyte function-associated antigen 3 and FcRII receptors (CD16) both before and after treatment with phosphatidylinositol-specific phospholipase C. The patient’s erythrocytes lacking DAF and CD59/MACIF expression corresponded to the proportion of complement-sensitive cells at the onset of acute leukemia. These DAF- and CD59/MACIF-deficient erythrocytes disappeared almost completely with progression of the leukemia. In conclusion, it appears that the expression of glycosylphosphatidylinositol-linked membrane proteins by leukemic cells was heterogeneous and discordant in our patient, and that the leukemic cells were derived from the PNH clone because of their deficiency of CD59/MACIF. It is also suggested that DAF could compete more effectively than CD59/MACIF for a limited number of anchor molecules available on the proliferating leukemic cells.

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PAROXYSMAL NOCTURNAL hemoglobinuria (PNH) is an acquired disorder characterized by increased susceptibility of erythrocytes to complement-mediated hemolysis. This unusual susceptibility to complement also appears to extend to the platelets and granulocytes of PNH patients. These facts suggest that PNH is a stem cell disorder since all the hematopoietic lineages are affected, and indicate that the abnormality must occur at least at the level of the pluripotent hematopoietic stem cell. Moreover, it has been reported that a decrease of progenitor cells and/or hypersensitivity of precursors to complement can be detected in PNH. The latter finding implies the increased destruction of PNH cells at the precursor level in the bone marrow. In rare cases, PNH transforms into acute leukemia, suggesting that it is related to the myelodysplastic disorders.

It has recently been reported that decay accelerating factor (DAF), a 70-Kd membrane protein involved in inhibition of the complement cascade at the level of the C3 and C5 convertases, is deficient in PNH cells, although it is normally expressed by most cells that come into contact with complement. Moreover, PNH erythrocytes have been shown to be deficient in anchor complement regulatory protein (ie, homologous restriction factor [HRF] or C8-binding protein [C8bp]), which is also widely distributed in blood cells. This protein inhibits formation of the membrane attack complex on autologous cells. More recently, Sugita et al reported another complement regulatory membrane protein, designated as membrane attack complex inhibitory factor (MACIF), which also inhibits membrane attack complex formation by preventing the binding of C9 to C5b-8. Despite having the same action as HRF/C8bp, MACIF is a structurally distinct protein, since the molecular weight of MACIF is 18 Kd while that of HRF is 65 Kd. MACIF is a glycosylphosphatidylinositol (GPI)-anchored membrane protein, which can be removed from the cell surface by phosphatidylinositol-specific phospholipase C (PIPLC) as is the case for DAF and HRF/C8bp. MACIF is reported to be the same protein as CD59, 20-Kd HRF, and membrane inhibitor of reactive lysis. It has been reported that CD59/MACIF is also deficient on PNH erythrocytes. Therefore, the defective expression of DAF, HRF/C8bp, and CD59/MACIF by PNH blood cells may lead to their increased susceptibility to complement-mediated hemolysis or destruction.

The development of acute nonlymphocytic leukemia in PNH patients is a rare occurrence, and only 21 such patients have been reported. We have also had two patients (one previously unreported) with this complication among our 76 patients with PNH. When leukemia occurs, the abnormal erythrocytes often disappear, suggesting that the patient’s marrow is replaced by a second abnormal leukemic clone. Recently, Devine et al reported a PNH patient who progressed to acute myeloblastic leukemia and had leukemic cells lacking DAF and neutrophil alkaline phosphatase. They suggested that these leukemic cells had been derived from the PNH clone.

This report describes the expression of DAF and CD59/MACIF on leukemic cells and erythrocytes from a PNH patient who developed acute myelocytic leukemia. In May 1990, the leukemic cells comprised 70% of the mononuclear cells in the bone marrow and 76% of those in the peripheral blood. They consisted of a mixture of positive and negative populations, including single DAF-positive cells. In August 1990, almost 100% of the peripheral mononuclear cells were leukemic blasts, and these consisted of a single population with reduced DAF expression. Single-color flow cytometric analysis showed that the leukemic cells lacked CD59/MACIF, while control leukemic cells (n = 3) expressed both DAF and CD59/MACIF. Leukemic blasts from this patient and six control patients expressed lymphocyte function-associated antigen 3 and FcRII receptors (CD16) both before and after treatment with phosphatidylinositol-specific phospholipase C. The patient’s erythrocytes lacking DAF and CD59/MACIF expression corresponded to the proportion of complement-sensitive cells at the onset of acute leukemia. These DAF- and CD59/MACIF-deficient erythrocytes disappeared almost completely with progression of the leukemia. In conclusion, it appears that the expression of glycosylphosphatidylinositol-linked membrane proteins by leukemic cells was heterogeneous and discordant in our patient, and that the leukemic cells were derived from the PNH clone because of their deficiency of CD59/MACIF. It is also suggested that DAF could compete more effectively than CD59/MACIF for a limited number of anchor molecules available on the proliferating leukemic cells.

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tient who developed acute myeloblastic leukemia, as determined by flow cytometry using anti-DAF and/or anti-CD59/MACIF monoclonal antibodies.

MATERIALS AND METHODS

Patient. The patient was a 64-year-old man diagnosed as having PNH on the basis of clinical and hematologic findings and a positive Ham's test in September 1985. He was intermittently treated with hydroxyurea for massive splenomegaly from August 1987. In May 1990, he was diagnosed as having acute myeloblastic leukemia with >30% blasts in the peripheral blood. Intensive chemotherapy could not be performed, because the patient had secondary myelofibrosis, severe pneumonia, and esophageal varices. He died of respiratory failure due to pleural effusion by leukemic infiltration and severe pneumonia on September 17, 1990. Peripheral blood or bone marrow samples from nine patients with acute myeloblastic leukemia were used as the control.

Cells. Peripheral blood and/or bone marrow cells (aspirated from the sternum with preservative-free heparin) were used. Erythrocytes were stored in Alsever's solution until use, and unreserved leukemic cells were also studied. Preserved leukemic cells were used only for immunofluorescence staining and flow cytometric analysis of cell surface DAF expression before and after PIPLC treatment. Mononuclear cells (MNC) were separated with Ficoll-isopaque (Pharmacia, Sweden) and used for flow cytometric one-color or two-color analysis. Some of these cells were subjected to CytoSpin preparation (Shandon, England), and the proportion of leukemic cells was determined.

Buffers. The buffers used were phosphate-buffered saline (PBS), pH 7.2, consisting of 10 mmol/L sodium phosphate, 150 mmol/L NaCl, and 0.02% sodium azide, as well as PBS with added 1% bovine serum albumin and 0.1% sodium azide instead of 0.02% sodium azide (PBS-BSA).

Anti-I antibody. Human anti-I antibody with a high agglutination titer (×16,384) was obtained from a patient with cold agglutinin disease. The serum was stored at -80°C. Just before the complement lysis sensitivity (CLS) test, the serum was thawed and the required dilution (×35) was prepared in veronal gelatin buffer.

Monoclonal antibodies. Three mouse monoclonal anti-DAF antibodies (Ia10, IiH6, and VIIIT)13 and a mouse monoclonal anti-CD59/MACIF antibody (3E1)25 (IgGl) were used. 3G8, a mouse monoclonal anti-CD59/MACIF antibody, was purchased from Immunotech S.A. (France). The CLS test developed by Rosse and Dacie was carried out in one-half scale. Peripheral blood samples from seven healthy volunteers were used as the control.

PIPLC treatment of cells. Leukemic cells (2 × 10⁶ cells/mL) were washed with PBS-BSA and incubated at 37°C for 1 hour with PBS containing 1 mg/mL BSA and 10 µg/mL PIPLC (Boehringer Mannheim GmbH, West Germany). Then the cells were washed and surface antigen (CD16, LFA-3, or DAF) analysis was performed by immunofluorescence flow cytometry. As a control, the cells were incubated under identical conditions without PIPLC.

Immunofluorescence staining and flow cytometric analysis of cell surface expression of DAF, CD59/MACIF, CD16, and LFA-3. Two-color staining analysis of anti-DAF and -CD59/MACIF antibodies was carried out by flow cytometry. First, erythrocytes or leukemic cells (2 × 10⁶ cells in 50 µL of PBS-BSA) were treated for 30 minutes on ice with 50 µL of a mixture of anti-CD59/MACIF monoclonal antibody (40 µg/mL in PBS-BSA) or an irrelevant monoclonal antibody of the same subclass as a control. Then the cells were washed three times, resuspended in 50 µL of PBS-BSA, and mixed with an equal volume of 1:10 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (H + L) (Tago, Inc., CA). After incubation for 30 minutes on ice and thorough washing, the cells were subjected to two-color staining. Normal mouse serum (50 µL) was then added to the resuspended cells (50 µL), and the mixture was incubated for 15 minutes on ice. The cells were then stained for DAF by incubation with 50 µL of a mixture of the bio-tinylated anti-DAF antibodies (10 µg/mL each)47 or of irrelevant monoclonal antibodies of the same subclasses, and reacted with 10 µL of phycoerythrin (PE)-conjugated streptavidin (Biomeda Corp, CA) for 30 minutes on ice. Cells were then analyzed using a FACSscan (Becton Dickinson, Mountain View, CA). The cells subjected to negative control staining were analyzed first to determine the four regions corresponding to positive for DAF only, positive for both antigens, negative for both, and positive for CD59/MACIF only (regions 1 to 4, respectively).

Single-color analysis of anti-DAF or anti-CD59/MACIF monoclonal antibody staining was also performed by flow cytometry on the same peripheral and/or bone marrow samples used for the two-color analysis described above.

Single-color analysis before and after PIPLC treatment was performed using anti-CD16 or -LFA-3 or -DAF monoclonal antibodies

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Table 1. Clinical and Laboratory Findings, Results of the CLS Test, and Two-Color Analysis of DAF and CD59/MACIF Expression by PNH Erythrocytes During the Clinical Course of Our Patient

Each fraction was defined as in Materials and Methods, and the actual analyses are shown in Fig 1.

Abbreviation: NT, not tested.

* Neg, Int, and Pos refer to the negative, intermediate and positive fractions, respectively.
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Fig 1. Flow cytometric analysis of DAF and CD59/MACIF expression by normal and PNH erythrocytes. In this two-color analysis, four regions (shown by the lines) were determined from the flow cytometric data of negative controls examined simultaneously. The expression of DAF (FL2) and CD59/MACIF (FL1) by PNH erythrocytes was defined as negative or positive according to the intensity of detection of the FITC (FL1)-labeled or PE (FL2)-labeled antibodies. (A) Normal controls (n = 31), (B) PNH patient (May 25, 1990), (C) PNH patient (June 20, 1990), (D) PNH patient (June 27, 1990), (E) PNH patient (July 3, 1990), (F) PNH patient (August 8, 1990). Expression of both antigens was positive on the erythrocytes of the 31 healthy volunteers.

RESULTS

CLS testing of PNH erythrocytes. CLS testing of PNH erythrocytes was carried out during the clinical course of the patient and the results are shown in Table 1. The erythrocytes examined consisted of PNH I and III cells, except for the specimen obtained on August 8, 1990, in which no complement-sensitive erythrocytes were found by the CLS test.

Cell surface DAF and/or CD59/MACIF expression. Two-color and single-color flow cytometric analysis was performed using anti-DAF and/or anti-CD59/MACIF monoclonal antibodies.

The results of the two-color analysis of erythrocytes are shown in Fig 1. At all examinations except that on August 8, 1990, the PNH erythrocytes of the patient included both positive and negative populations. On August 8, the patient's erythrocytes were all positive. On the other hand, the erythrocytes from normal volunteers were always double positive. The percentages of each population are shown in Table 1.

Single-color analysis of MNC from the patient's peripheral blood and/or bone marrow was performed on May 25 and August 8, 1990 (Fig 2). Both the peripheral blood and bone marrow MNC included double-positive, double-negative, and DAF-positive populations in May 1990. In August 1990, the peripheral MNC were generally DAF-positive or double-negative, with less double-positive cells. Single-color analysis of MNC in August 1990 showed that almost all cells had the same weak intensity of CD59/MACIF.
expression as the negative control, and that DAF expression was also lower than in the control patients (Fig 2). On the other hand, the leukemic cells from three control patients with acute leukemia were all double positive. The percentage of cells in each region on flow cytometric analysis was calculated and the results are shown in Table 2. The proportion of leukemic cells in the PNH and control MNC samples is also shown in Table 2.

Expression of cell surface CD16, LFA-3, or DAF by leukemic cells before and after PIPLC treatment. Single-color flow cytometric analysis using anti-CD16, anti-LFA-3, and anti-DAF monoclonal antibodies was performed on the leukemic cells of the PNH patient (August 1990) and eight control leukemia patients before and after PIPLC treatment of the cells.

All three surface antigens were expressed on the leukemic cells of the PNH patient and the control leukemia patients before PIPLC treatment, although expression of CD16 was only weakly positive in all cases (Fig 3). The expression of CD16 and LFA-3 was not affected by PIPLC treatment, but DAF expression was decreased after PIPLC treatment in all cases (Fig 3).

The leukemic cell samples of the PNH patient examined for LFA-3 and CD16 were the same as those used for two-color analysis of DAF and CD59/MACIF (Table 2). In the control patients with acute myelocytic leukemia, the percentage of leukemic cells among MNC was respectively 96%, 81%, and 99% in the examination for LFA-3, 79%, 96%, and 99% in the analysis of CD16, and 70%, 93%, and 90% in the DAF analysis.

DISCUSSION

The leukemic cells of our PNH patient were characteristically myeloblastic (M1 in the French-American-British classification) and their expression of DAF and CD59/MACIF was heterogeneous. In May 1990, the bone marrow and peripheral blood MNC consisted of both positive and negative populations, including single DAF-positive cells. On
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...the PNH clone because of their lack of CD59/MACIF. Devine et al have previously reported DAF-negative leukemic cells which possessed both DAF and CD59/MACIF. These findings indicate that expression of DAF and CD59/MACIF by the leukemic cells of the PNH patient was heterogeneous, and also suggest that the leukemic cells were derived from the PNH clone because of their lack of CD59/MACIF. Devine et al have previously reported DAF-negative leukemic cells in PNH, but they did not investigate the expression of CD59/MACIF. The discordant expression of DAF, CD59, and CD16 by PNH polymorphonuclear leukocytes and monocytes, and the heterogeneous expression of DAF and CD59/MACIF by the various phenotypes of PNH erythrocytes (I, II, and III) have also been reported.46 In our study, we also found that on leukemic cells in PNH, the expression of GPI-anchored membrane proteins was discordant.

The mechanism accounting for the apparently unrelated multiple defects in blood cells from PNH patients has become clarified by findings indicating that DAF, HRF/C8bp, and CD59/MACIF all belong to a newly characterized group of membrane proteins.24,31-37 These proteins share the property of being anchored to the cell membrane by glycaninositol-phospholipids, which are covalently bound to the C-terminal residue of the peptide. Therefore, it is conceivable that abnormal cells in PNH patients have a defect in either the synthesis of this glycolipid structure or in the mechanism attaching the glycolipid to the peptide.30,50 However, in our present patient the expression of CD59/MACIF was almost completely deficient on the leukemic cells, while that of DAF was only slightly reduced (Fig 2). The similar expression of both proteins on blood and somatic cells was reported in a patient with apparently autosomal recessive inheritance of complete deficiency 20-Kd HRF.52 This fact suggests that the mechanism of the CD59/MACIF deficiency was fundamentally different in the two patients. Recently, Mahoney et al showed that PNH granulocytes did not synthesize detectable amounts of the complete GPI,13 and Thomas et al reported the differential transfer of available GPI cores to proteins containing GPI-anchor attachment sequences in a murine T cell hybridoma mutant with limited production of glycolipid cores.54 These observations suggest the interpretation that the GPI-anchor deficiency was not complete in our patient, and that DAF was able to compete more effectively than CD59/MACIF for the limited number of anchor molecules available on the leukemic cell membranes. This concept provides a possible explanation of why the proportion of leukemic cells among MNC was 76% when the proportion of positive cells was only 65.3% (Table 2): the synthesis of GPI-anchor membrane proteins was sufficient at the incipient stage of leukemic transformation, but was inadequate following the progression to acute leukemia. Of course, it is also possible that a leukemic clone proliferated that was constitutionally deficient in CD59/MACIF expression. However, this would not explain the finding described above, because in this case the proportion of leukemic cells must be theoretically equal to the complement-sensitive population of DAF- and CD59/MACIF-deficient cells at the onset of leukemia.

The CLS test is a useful and standard means of determining the phenotype of PNH erythrocytes.52,60 In our present patient, the CLS test was performed nine times over 5 years. During this period, the PNH erythrocytes of the patient constantly showed the PNH I and III phenotypes, except in the last test, and the proportion of PNH III erythrocytes was 20% to 40%. However, after the onset of acute leukemia, the proportion of PNH III erythrocytes showed a marked decrease and they finally disappeared. This disappearance of PNH III erythrocytes might have been due to the rapid proliferation of the leukemic subclone as well as to the influence of transfusion. After transfusion, 1.4% of the patient’s erythrocytes were in Fraction 3 on flow cytometry (Table 1), while the proportion of cells in Fraction 3 was <1% in 31 normal controls. These findings may suggest the persistence of a few red blood cells derived from the original PNH clone in August 1990.

In an attempt to clarify the discordant expression of DAF and CD59/MACIF by our patient’s leukemic cells, we also studied the expression of two other GPI-anchored membrane proteins, CD16 and LFA-3.5,6,61 LFA-3 is a cell surface glycoprotein of 45 to 70 Kd that is widely distributed on both hematopoietic and nonhematopoietic cells and is deficient in PNH cells.58-61 It has been reported that LFA-3 is present in two forms on white blood cells, a phosphatidylinositol-linked form and a distinct hydrophobic polypeptide chain-linked form.61 In the present study, we found that LFA-3 was expressed by the myeloblasts of this patient before PIPLC treatment and was not affected by PIPLC. Myeloblasts from three control patients with acute leukemia showed the same

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Abbreviations: PB, peripheral blood; MNC, mononuclear cells; BM, bone marrow.

*Region, as defined in the text and shown in Fig 2.
pattern of LFA-3 expression. These findings suggest that myeloblast LFA-3 was the hydrophobic polypeptide chain-linked form and not the phosphatidylinositol-linked form. CD16 is a low-affinity receptor for the Fc domain of human IgG on human leucocytes, and is also deficient in PNH cells. On granulocytes, CD16 is linked to the membrane by a phosphatidylinositol-glycan anchor. It is a 50 to 80 Kd glycoprotein and a late differentiation antigen that is first expressed at the metamyelocyte stage. Myeloblasts from our patient showed weak expression of CD16 before PIPLC treatment, which was not affected by PIPLC; leukemic cells from the three control patients with acute leukemia also showed the same result. These findings suggest that weak CD16 expression on the membrane surface of myeloblasts may occur in other (unknown) forms besides the GPI-linked form. In addition, the expression of DAF by leukemic cells was decreased after PIPLC treatment in both our PNH patient and the control patients with acute myeloblastic leukemia (Fig 3). However, we were unfortunately unable to reconfirm the expression of other GPI-anchored membrane proteins besides DAF and CD59/MACIF by the leukemic cells of this patient.

In conclusion, it appears that the expression of GPI-anchored membrane proteins by leukemic cells was heterogeneous and discordant in our PNH patient, and that the leukemic cells were derived from a PNH clone because of their lack of CD59/MACIF expression. In addition, it seems that DAF may have been able to compete more effectively than CD59/MACIF for a limited number of anchor molecules available on the membranes of the proliferating leukemic cells.

ACKNOWLEDGMENT

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Discordant and heterogeneous expression of GPI-anchored membrane proteins on leukemic cells in a patient with paroxysmal nocturnal hemoglobinuria

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