Development of the Glycosylphosphatitylinositol-Anchoring Defect Characteristic for Paroxysmal Nocturnal Hemoglobinuria in Patients With Aplastic Anemia

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The introduction of immunosuppressive therapy for treatment of aplastic anemia has led to a considerable improvement in the prognosis of this disease. However, long-term follow-up of these patients showed a high incidence of "late" hematologic complications such as myelodysplasia and paroxysmal nocturnal hemoglobinuria (PNH). The detection of the glycosylphosphatitylinositol (GPI)-anchoring defect on peripheral blood cells of patients with aplastic anemia is now available as a new tool for early specific detection of PNH and is more sensitive than the Ham-test. Granulocytes appear to be the first cells affected in 11 patients with a GPI-anchoring defect of 29 suffering from aplastic anemia investigated in the present study. The later involvement of erythrocytes and a positive Ham test was observed in 1 patient. From our data it can be concluded that the rate of PNH resulting from aplastic anemia might be higher than reported in the literature when the Ham test alone was used for follow-up. Furthermore, our results suggest the clinical response to immunosuppressive therapy appears to be worse in the group developing the GPI-anchoring defect than in the group without this deficiency.

PAROXYSMAL nocturnal hemoglobinuria (PNH) arises as a common "late" complication in patients with aplastic anemia (AA).[1-4] This has become evident since immunosuppression has been established as a successful therapy.[5,6] PNH has recently been characterized as an acquired disorder in which subpopulations of peripheral blood cells deficient for glycosylphosphatitylinositol (GPI)-anchored surface molecules appear in the circulation.[7-9] These abnormal cells arise as a consequence of expansion of one or more bone marrow progenitors altered by somatic mutation.[10] The absence of GPI-anchored complement-regulating proteins such as decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) leads to an abnormal susceptibility of deficient cells to autologous complement activation, resulting in the classical symptoms of the disease such as intravascular hemolysis and cell death.[9,11,12] In addition, the abnormally high incidence of venous thromboses appears to be associated with complement activation.[13,14] This defect can be present on all leukocytes and was observed on 5% to 30% of lymphocytes from patients with classical PNH.[15,16] Recently, the GPI-anchoring defect has been identified biochemically using continuously growing lymphocyte cell lines. The defect appears to be homogenously localized in the transfer of N-acetyl-glucosamine (GlcNAc) from UDP to phosphatidylinositol (PI) and corresponds to that found in Thy-1$^-$ mouse lymphoma mutants of the complementation class A.[17,18]

In earlier studies, the diagnosis of a clonal disease such as PNH in AA was based on a positive Ham test that is confined to the affection of erythrocytes. Previously, we and others have shown that the cytofluorometric analysis of peripheral blood cells with respect to their surface expression of GPI-anchored proteins is a more specific and sensitive method for establishing the diagnosis of PNH, especially in those patients with no or minor involvement of red blood cells but with affected granulocytes and monocytes.[16,19]

Here, we used this method to follow-up patients with AA. We found that affected leukocytes could be detected more frequently and far earlier than affected erythrocytes. Involvement of the latter coincided with a positive conventional Ham test. In addition, a poor clinical response to immunosuppressive therapy was observed more frequently in the group of patients in which GPI-deficient cells were detected.

MATERIALS AND METHODS

Patients. Twenty-nine patients (16 males and 13 females; age range, 18 to 65 years; mean age, 43; time after immunosuppressive treatment: range, 2 to 128 months; mean, 32 months) with aquired idiopathic AA, including 1 with posthepatitic aplasia, were investigated every 6 months. Twenty were diagnosed as severe AA (SAA) and 9 as moderate AA (MAA) based on the criteria of the International Aplastic Anemia Study Group (IAASG 1987). Two patients died from cerebral bleeding.

Twenty-two patients were treated with a triple-drug therapy consisting of antilymphocyte globulin (ALG; 0.75 mL/kg [days 1 through 8] applied intravenously over 8 hours), methylprednisolone (MP; 5 mg/kg [days 1 through 8], 1 mg/kg [days 9 through 14], and thereafter continuously reduced) and cyclosporine A (CsA; 6 mg/kg twice per day or adapted to a plasma level of 100 to 200 ng/mL, for at least 3 months).[5] One patient was treated with the same regimen under exclusion of CsA. Four patients received, in addition to ALG, MP, and CsA, interleukin-3 (IL-3; 250 $\mu g/m^2/d$ subcutaneously for 3 months) as second line protocol after relapse or lack of response.[6] Two patients received a combination of ALG/MP with androgenes (Andro) administered as metenolon-acetate (3 mg/kg/d for at least 3 months).[20]

Cell preparation. Heparinized blood samples were obtained by

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venous puncture and then mixed with an equal amount of Hanks' Balanced Salt Solution (HBSS). Peripheral blood mononuclear cells (PBMC) were harvested by Ficoll density centrifugation. Granulocytes and erythrocytes were obtained from Ficoll pellets and were further separated by hydroxyethylstarch. Contaminating erythrocytes in either the mononuclear or the granulocyte fraction were removed by hypotonic lysis.

Acid hemolysis test (Ham test). The Ham test was performed according to the standard protocol. Briefly, patient's erythrocytes were exposed to acidified normal serum (pH 6.5 to 7.0) for 1 hour at 37°C. Tests with more than 2% lysis were considered positive.

Monoclonal antibodies (MoAbs). MoAbs recognizing GPI-anchored surface molecules were used as described previously.[10] CD14 MoAb Mo2 was kindly provided by Dr R. Todd (Simpson Memorial Research Institute, Ann Arbor, MI). CD16 MoAb 3G8 was purchased from Medarex (West Lebanon, NH). CD48 MoAbs J4.57 and MEM102 were a gift of Dr J. Pesando (Biomembrane Institute, Seattle, WA) and Dr V. Horejsi (Czechoslovak Academy of Sciences, Prague, Czech Republic), respectively. CD55 anti-DAF MoAbs BRIC 110 and 134-30 were obtained from Dr J. Anstee (South Western Regional Transfusion Centre, Bristol, UK) and Dr R. Vilella (Hospital Clinic, Barcelona, Spain), respectively. CD58 anti–LFA-3 MoAb TS/2-9 was kindly provided by Dr T.A. Springer (Harvard Medical School, Boston, MA) and CD59 MoAb MEM43 was a generous gift of Dr V. Horejsi (Prague, Czech Republic).

In addition to antibodies recognizing GPI-anchored determinants, CD64 anti-FcRγI MoAb 32.2 was purchased from Medarex. Control MoAb W6/32 recognizes the monomorphic determinant of HLA class I molecules. CD44 MoAb BRIC35 was obtained from Dr J. Anstee (Bristol, UK). Fluorescein isothiocyanate-conjugated goat-antimouse $F(ab')_2Ig$ (GM-FITC) antiserum was purchased from Dianova (Hamburg, Germany).

Immunofluorescence studies. Indirect immunofluorescence was performed with GM-FITC as described.[16,21] The washing buffer was phosphate-buffered saline containing 0.1% bovine serum albumin (PBS/BSA). Samples were then analyzed on a fluorescence-activated cell sorter FACScan (Becton Dickinson, Heidelberg, Germany). Using forward and site scatter gating, peripheral blood lymphocytes (PBL) and monocytes could be analyzed separately from the PBMC fraction. Ten thousand cells were analyzed in each sample. Fluorescence intensity was presented on a 4 log scale as histograms. A proportion of at least 5% GPI-negative cells was considered significant for deficient cells in each fraction.

RESULTS

Early detection of GPI-deficient peripheral blood cells in AA patients. As described in a previous study, leukocytes and erythrocytes with GPI deficiency can be detected by immunofluorescence using MoAbs against a series of GPI-anchored antigens.[16] In addition to CD16, CD55 and CD59 antibodies are also needed for the analysis of granulocytes because $CD16^-$ granulocytes might also represent eosinophils. Furthermore, in some AA patients without detectable GPI-anchoring defect at the time of diagnosis, the defect can be observed on a significant number of granulocytes in follow-up studies without any involvement of erythrocytes and without a positive classical PNH test. Figure 1 shows affected granulocytes that are negative for CD16, CD55, and CD59 from 3 representative AA patients. During the whole observation period these deficient cells do not appear transiently.

AA patients with GPI-deficient leukocytes subsequently develop affected erythrocytes and a positive acid hemolysis test follows. In our study, almost all patients with a GPI-anchoring defect on PMN or monocytes had normal erythrocytes and also a negative Ham test. Once developed, deficient cells remained constantly detectable in all patients of our study. One female patient had an interesting follow-up period. Severe AA was first diagnosed in June 1984; treatment with ALG/MP/androgenes was followed by a complete remission. Her first relapse occurred in February 1985, and a second course of ALG/MP resulted in a second complete remission. A second relapse occurred in November 1985, and monotherapy with CsA was followed by complete remission that was initially CsA-dependent. Despite continuation of CSA therapy, a third relapse was observed in March 1989; treatment with ALG/MP/CsA again resulted in complete remission. During the whole observation period, Ham tests were negative. In September 1990, she relapsed again and, for the first time, a defect of GPI-anchored molecules was diagnosed on granulocytes and monocytes. One could speculate that the shoulder shown on cytofluorographic analysis of erythrocytes using CD59 MoAbs might correspond to cells already affected at that time. Nevertheless, the Ham test was negative at that time. Later, the defect was also detectable on erythrocytes corresponding to a positive Ham test (Fig 2). After treatment with ALG/MP and CsA in combination with IL-3, complete remission was once again achieved.

The presence of a GPI-anchoring defect coincides with a relapse after complete response in some patients. One patient (U.G.) with severe AA first responded well after triple combination therapy, and GPI-deficient cells were not present at time of diagnosis and again 12 months later. After a period of 18 months, he developed a relapse. At the same time, GPI-deficient granulocytes and monocytes were detected in a proportion of 23% and 10%, respectively. Figure 3 shows granulocytes affected after 18 months. Another patient (E.P., not shown) had a complete remission after triple immunosuppressive regimen. CsA was then administered for 9 months. After discontinuation of CsA treatment, he developed a first relapse. Partial remission was achieved after a second course in combination with IL-3. One year later, he relapsed a second time; a third course of triple combination treatment was unsuccessful. In parallel to the second relapse, he developed GPI-deficient PMN.

Follow-up of AA patients suggests that detectable GPI-deficient cells are associated with a worse clinical response after immunosuppressive therapy. In our study, 12 patients developed a GPI-anchoring defect on PMN and monocytes; one of these also developed a GPI-anchoring defect on erythrocytes. All patients developed the defect within a range of 3 months to 10 years during the course of the disease. In none of them was the Ham test significantly positive when the defect was already present on neutrophils and monocytes. In 2 patients the first diagnosis coincided with detection of the GPI-anchoring defect. Of the 17 patients without a defect, 13 achieved complete remission, with 2 patients being CsA-dependent. In this group, 7 of 17 patients had a mild form of the disease. Partial or no remission was observed in 3

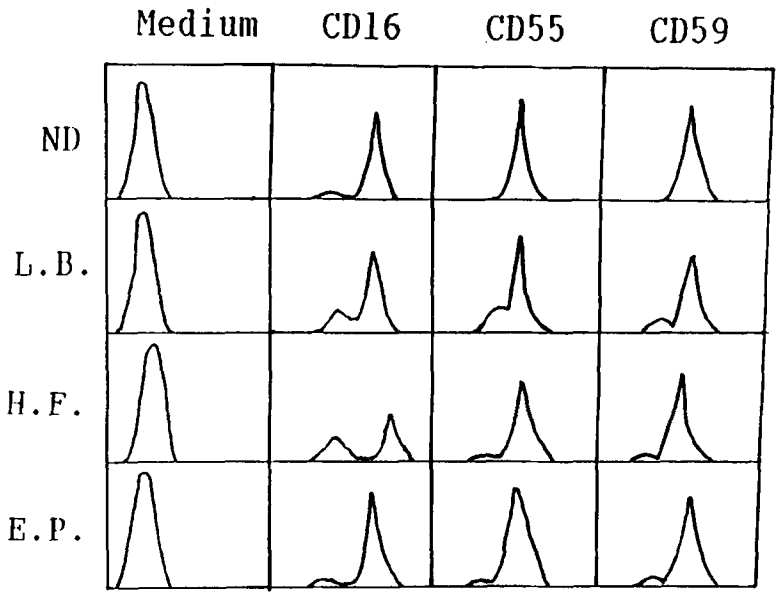

Fig 1. Cytofluorographic analysis of granulocytes from 1 normal donor (ND) and 3 representative AA patients (L.B., H.F., and E.P.) with granulocytes demonstrating deficient expression of GPI-anchored CD16, CD55, and CD59 antigens.

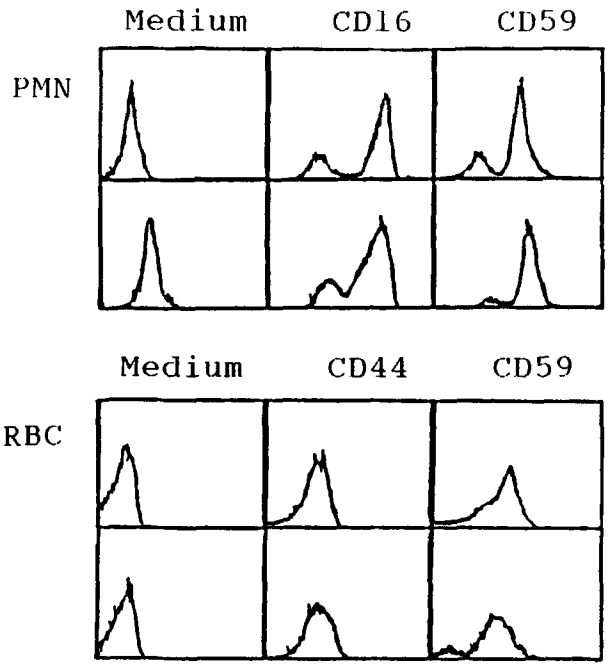

Fig 2. Cytofluorographic analysis of granulocytes and erythrocytes of 1 AA patient (A.Z.) for GPI-anchored surface molecules (CD16 for granulocytes; CD59 for granulocytes and erythrocytes) at two different points in time. CD44 MoAbs were used to control the recognition of a transmembranous surface molecule in the same cells. (Upper row) Analysis at the first detection of GPI-anchoring defect. (Lower row) Analysis at the fourth relapse when the Ham test became positive for the first time.

patients of this group, respectively; however, except for 1 patient, this was only observed in patients with mild disease (Table 1). In contrast, in the group of patients with GPI deficiency (2 with mild and 10 with severe disease), only 3 responded with complete remission after therapy. Two patients had a partial remission and 6 finally did not respond (Table 2). The association of clinical response, ie, less than a complete response and acquisition of the GPI-anchoring defect, did achieve statistical significance using Fisher's exact test ($P < .01$). In contrast, the relation of clinical response and severity of the disease was not statistically significant ($P = .3525$).

DISCUSSION

Clonal diseases such as myelodysplasia and PNH have been described by several investigators as late hematologic

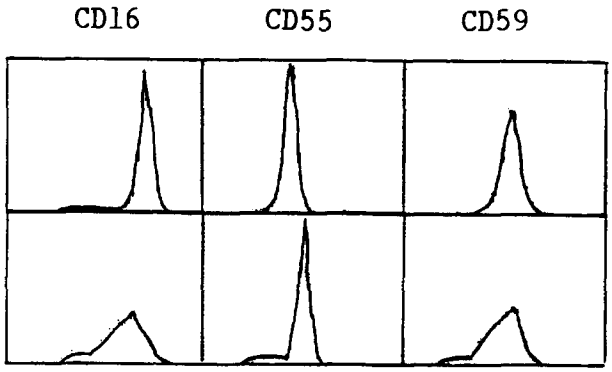

Fig 3. Cytofluorographic analysis of granulocytes for GPI-anchored antigens from 1 AA patient (U.G.). (Upper row) Analysis during complete remission after immunosuppressive treatment. (Lower row) Analysis shortly after relapse.

Table 1. AA Patients Without GPI-Anchoring Defect

Patient	Severity	Therapy	Time*	Response
M.A.	MAA	ALG, MP, CsA	6	CR
A.B.	SAA	ALG, MP, Andro	115	CR
U.C.	MAA	ALG, MP, CsA	12	NR
D.G.	SAA	ALG, MP, CsA	46	PR
G.G.	SAA	ALG, MP, CsA	50	CR
B.H.	SAA	ALG, MP, CsA	16	CR
J.H.	SAA	ALG, MP, CsA	17	CR
G.H.	SAA	ALG, MP	60	CR
K.H.	MAA	ALG, MP, CsA	22	PR
E.K.	MAA	ALG, MP, CsA	28	NR
R.L.	SAA	ALG, MP, CsA	74	CR
G.R.	MAA	ALG, MP, CsA	12	CR
C.S.	SAA	ALG, MP, CsA	15	CR
B.S.	MAA	ALG, MP, CsA-CsA-Monoth	50	CR
A.S.	SAA	ALG, MP, CsA	6	CR
H.S.	SAA	ALG, MP, CsA-CsA-Monoth	19	CR
HJ.S.	MAA	ALG, MP, CsA	128	CR

Abbreviations: CsA-Monoth, continuation of CsA monotherapy due to CsA dependency; NR, no response; PR, partial response; CR, complete response.

* Total observation time in months.

complications of AA.[1-3] Recently, we and others have developed a cytofluorometric test for early phenotypic detection of peripheral blood cells for their missing expression of GPI-anchored surface molecules.[16,19] We present here for the first time data on the follow-up of AA patients whose blood has been cytofluorographically analyzed for this defect.

Within an observation period of 3 years, 29 patients with AA (mean 43 months after immunosuppressive therapy) were investigated at 6-month intervals. We found that the GPI-anchoring defect on leukocytes characteristic for PNH is not observed in healthy people, but can be detected quite early in 36% of all AA patients. In 1 patient, overt disease with intravascular hemolysis and a positive Ham test resulted 7 years after the first diagnosis and 1 year after the first detection of GPI-deficient granulocytes. In 2 other patients, we found that early relapse within 18 months after initial complete remission was associated with the appearance of GPI-deficient granulocytes and monocytes in the circulation. In addition, the clinical response to immunosuppressive therapy appears to be worse in the group of patients who developed the defect. This association was statistically significant. In contrast, the relation of therapeutic response and severity of the disease did not achieve statistical significance.

Cytofluorographic analysis for detection of GPI-deficiency appears to be highly advantageous compared with conventional laboratory tests that are confined to detecting affected erythrocytes.[16,19] In our studies, only 1 patient has developed a positive acid hemolysis test. We therefore suggest that cytofluorographic analysis might lead to earlier detection of PNH. Presumably, patients with AA may acquire PNH as a late hematologic complication after immunosuppressive therapy at a higher rate than described in the literature.[1-4] This hypothesis could explain the survival curves in which no survival plateau occurred after an observation period of 8 years.[22]

Although AA may be a heterogeneous disease in which multiple factors play a role, this association supports the assumption of damage to the hematopoietic stem cell in at least some cases with AA. This has been suggested in previous reports investigating long-term bone marrow cultures in normal and AA patients using crossover experiences.[23]

The relationship between PNH and AA still remains unclear. In our recent studies, we have elucidated the biochemical basis leading to GPI deficiency in PNH. The defect has recently been identified as lack of transfer of GlcNAc to phosphatidylinositol in all patients.[18] So far, it is not clear exactly how the mutated stem cells arise and why they expand. It has been described that, in some AA patients, a monoclonal hematopoietic pattern can be detected.[24] One possible explanation would be the continuation of only a limited number of stem cells.[25] The presence of GPI-deficient cells could also alter the interaction of stem cells with their

Table 2. AA Patients With Development of a GPI-Anchoring Defect

Patient	Severity	Therapy	% Defective PMN	% Defective Monocytes	Time*	Response
M.A.	SAA	ALG, MP, CsA	9.5	7.2	3/9	NR
L.B.	SAA	ALG, MP, CsA	24	13	6/10	PR
M.D.	MAA	ALG, Andro	5.5	8.5	72/72	PR
W.D.	SAA	ALG, MP, CsA, IL-3	5.4	12.6	5/21	NR
H.Fl	SAA	ALG, MP, CsA	18.5	5.0	1/7	NR
H.Fu	MAA	ALG, MP, CsA	9.0	8.2	14/28	NR
U.G.	SAA	ALG, MP, CsA	5.7	8.1	19/25	PR
S.H.	SAA	ALG, MP, CsA	6.4	8.1	23/25	CR
S.I.	SAA	ALG, MP, CsA	15	n.d.	47/47	NR
R.K.	SAA	ALG, MP, CsA	7.8	11.9	1/24	CR
E.P.	SAA	ALG, MP, CsA, IL-3	9.5	9.2	25/36	PR
A.Z.	SAA	ALG, MP, CsA, IL-3	17.6	19.2	89/101	CR

All patients except A.Z. had a negative Ham test and did not exhibit GPI-deficient erythrocytes. A.Z. had 19.9% deficient erythrocytes and a positive Ham test.

* Time between first diagnosis and first detection of the defect in months/total observation time.

microenvironment. The requirement of GPI-anchored surface molecules for sufficient cell/cell communication and activation has been analyzed by using lymphocytes or PMN obtained from PNH patients, or by using lymphoma mutants deficient for GPI-anchored surface molecules.[26-29] Alternatively, enhanced complement susceptibility of affected bone marrow precursor cells has been discussed.[30]

The time when the alteration of bone marrow stem cells occurs remains unclear. The defect may be present at the time of diagnosis, but may not be detectable. Even cytofluorographic analysis has a limited sensitivity and at least 5% of cells in the circulation need to be affected. Recently, cDNA coding for the missing enzyme in the deficient cells has been cloned.[31] In addition, the corresponding gene has been localized to the X-chromosome. It has also been found that different mutations within the coding region lead to altered gene products at analysis of GPI-deficient cells from different PNH patients.[32] Further analysis, especially with respect to GPI deficiency in AA, might lead to the development of instruments for earlier detection of deficient cells in the future.

In summary, the detection of the GPI-anchoring defect on peripheral blood cells of AA patients after immunosuppressive therapy is a useful method for detecting late hematologic complications such as PNH. Furthermore, prospective long-term follow-up studies are needed to obtain more information on the relationship between these two disorders.

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