Expression of Recombinant Transmembrane CD59 in Paroxysmal Nocturnal Hemoglobinuria B Cells Confers Resistance to Human Complement

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic disorder characterized by complement-mediated hemolytic anemia, pancytopenia, and venous thrombosis. These clinical manifestations arise from an underlying molecular defect of bone marrow stem cells. Specifically, somatic mutations in the phosphatidylinositol glycan class A (PIG-A) gene result in the inability of blood cells to anchor complement-regulatory proteins (CD59 and DAF) to the cell surface via glycosyl phosphatidylinositol (GPI). In an attempt to circumvent the functional defect in PNH cells, a recombinant transmembrane form of CD59 (CD59-TM) was analyzed for the ability to regulate complement activity. Balb/3T3 stable transfectants expressing similar levels of either CD59-TM or native CD59 (CD59-GPI) were equally protected against human complement-mediated membrane damage. Treatment of these cells with phosphatidylinositol-specific phospholipase C failed to release CD59-TM from the cell surface.

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematopoietic disorder characterized clinically by hemolytic anemia and intravascular thrombosis resulting from an increased sensitivity of erythrocytes and platelets to complement.1,2 The disease manifestations have been attributed to the clonal expansion of a pluripotent stem cell population that is unable to express glycosyl phosphatidylinositol (GPI)-anchored complement regulatory proteins on the cell surface.3 The recent characterization of PNH cell lines derived from several different patients shows that the defect occurs in the first step of GPI-anchor synthesis involving the transfer of N-acetylgalactosamine (GlcNAc) to a phosphatidylinositol (PI) acceptor.4,5 More recently, replacement of the phosphatidylinositol glycan class A (PIG-A) gene, which participates in this transfer, has been shown to correct the GPI-anchoring defect in PNH B-cell lines from several different patients. Molecular characterization of these cells has shown that the somatic mutation is restricted to the PIG-A gene, but that the mutation differs between patients.6

The clinical manifestations of PNH have been attributed to the inability to regulate the lytic and cell-stimulatory activity of complement on the surface of erythrocytes and platelets.2,6 The cytolytic activity of human complement is mediated by the assembly of five proteins (C5b, C6, C7, C8, and C9) into a functional membrane attack complex, C5b-9.10,11 When assembled on the cell surface, the C5b-9 complex forms stable pores in the membrane, thereby altering the ion permeability of the cell. This can lead to cell lysis or, in some cases, sublytic activation.12,13 The hemolytic anemia associated with PNH can be attributed to increased C5b-9 deposition on erythrocytes.6 Additional studies have shown that thrombosis seen in PNH patients may be caused by increased C5b-9 deposition on platelets, which leads to procoagulant responses such as increased prothrombinase activity.2,14-16 Normally, human blood cells are protected against autologous complement activation by membrane proteins that block the assembly of functional complement pores. Two such proteins, DAF and CD59, are tethered to the membrane via GPI-anchors and both have been shown to be absent from erythrocytes and platelets, as well as other cell types, derived from PNH patients.17-19 DAF regulates complement activation by inhibiting C3 convertase,20 whereas CD59 inhibits the assembly of C5b-9 by interacting with C8 and C9.17,18 Evidence for the individual contributions of DAF and CD59 to the clinical symptoms associated with PNH have recently been described. Patients deficient in surface expression of DAF (Inb phenotype), but expressing normal levels of CD59, show no clinical signs of hemolytic disease or thrombosis.21,22 However, a patient genetically deficient in the expression of functional CD59 but expressing normal levels of DAF exhibits symptoms indistinguishable from PNH.23,24 In vitro studies have supported these results because isolated erythrocytes with the Inb phenotype resist complement-mediated lysis initiated through the alternative pathway.25 Additionally, purified CD59 incorporated into the membrane of the type III PNH erythrocyte protects these cells from complement damage.26,27 The type III PNH erythrocyte is highly sensitive to C5b-9-mediated lysis and is primarily responsible for the hemolytic anemia characteristic of PNH patients.26,27

In this study, we have used a recombinant, transmembrane form of CD59 (CD59-TM) generated by replacing the GPI-anchoring signal of CD59 with the carboxy-terminus of the

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membrane cofactor protein (MCP), a known transmembrane-anchored protein. The complement regulatory activity of CD59-TM expressed in BALB/3T3 cells was compared with that of wild-type CD59 (CD59-GPI). CD59-TM was successfully expressed on the surface of GPI-anchoring deficient cell lines, including PNH cells, and functioned to protect these cells from complement-mediated membrane damage.

**MATERIALS AND METHODS**

*CD59 cDNA constructs.* A transmembrane form of HuCD59 (CD59-TM) was constructed by replacing the putative GPI-anchor signal of CD59\(^{30}\) with the carboxy-terminal domain of the MCP. CD59 was truncated at amino acid 77 of the mature protein (CD59\(^{77}\)). These oligonucleotides amplified a fragment coding for amino acids 270-350 of the MCP mature protein, a region shown previously to comprise a functional transmembrane domain.\(^{31}\) An endogenous Su1 site found at the 3\(^{\prime}\) end of the fragment was used to ligate this domain to the 3\(^{\prime}\) end of CD59\(^{77}\), in the eukaryotic expression vector pCDNA 3 (Invitrogen, San Diego, CA). Full-length CD59 containing the GPI-anchor signal (CD59-GPI) was cloned into pCDNA 3, as described elsewhere.\(^{29}\) CD59-GPI and CD59-TM were further subcloned as BamHI fragments into the retroviral vector pLXSN.\(^{32}\)

**Amphotropic virus production.** Amphotropic virus was produced through an intermediate ecotropic packaging cell line as previously described.\(^{33}\) Briefly, y2 cells were transfected with LXSN containing CD59-TM using dimethyl sulfoxide shock followed by selection in D10 medium (Dulbecco's modified Eagle's medium with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin) containing G418 (500 mg/mL, active concentration). Transfectants were pooled and a 24-hour supernatant was harvested from cells at 90% confluency. The ecotropic virus stock was used to transduce the amphotropic packaging cell line PA317, which was also selected in 500 mg/mL G418 and a virus stock collected as described above. Amphotropic viral stocks of LXSN containing wild-type CD59 (CD59-GPI) or LXSN alone were obtained using similar methods.

**Balb/3T3-cell, mouse L-cell, and PNH B-cell stable lines.** Balb/3T3 cells (ATCC CCL 163; American Type Culture Collection [ATCC], Rockville, MD) were stably transfected with pLXSN containing CD59-GPI, CD59-TM, or vector alone as assessed by the presence or retention of the cytoplasmic indicator dye, calcein AM (Molecular Probes, Inc, Eugene, OR). Balb/3T3 cells and L cells were grown to confluence in 96-well plates, washed twice with Hank's Balanced Salt Solution (HBSS) containing 1% bovine serum albumin (BSA) and incubated with 10 mmol/L calcein AM at 37°C for 30 minutes. Cells were again washed twice before the addition of polyclonal anti-Balb/3T3 IgG (2 mg/mL) for 30 minutes of incubation at 23°C. After washing away unbound IgG, the complement components C5b, 6, and 7 were deposited by adding 20% C8-deficient human serum (Quidel, San Diego, CA) for 30 minutes of incubation at 37°C. Finally, cells were washed before adding purified C8/C9 (Quidel) at various concentrations for 30 minutes of incubation at 37°C. Dye release was measured on a Milipore (Philadelphia, PA) Cytofluor 2350 plate reader (excitation, 490 nm; emission, 530 nm). The percentage of dye release was calculated relative to total cell-associated dye determined from cells treated with 1% SDS. Dye release from cells not subjected to serum allowed the determination of background fluorescence and non-specific dye release. Dye release in this assay is a measure of sublytic membrane damage as trypan blue exclusion of cells after exposure to complement showed less than 2% cell lysis (data not shown).

**Complement-mediated membrane damage of PNH B cells was measured in a fluid phase assay.** Cells (5 \times 10^5) were washed three times with GVBSS\(^{++}\) (Sigma) before loading with 10 mmol/L calcein AM at 37°C for 30 minutes. Classical complement activation was initiated by the addition of anti-HLA Ab W6/32 (Accurate Chemical and Scientific Corp, Westbury, NY) at 10 mmol/L for 15 minutes of incubation at room temperature. After washing three times, cells were incubated with human C8-deficient serum plus purified C8 (Quidel) at various concentrations. Finally, cells were resuspended to 1 mL of PBS and dye release measured by FACS based on the ratio of intact cells retaining dye relative to cells incubated in 0% serum.
RESULTS

Analysis of CD59-GPI and CD59-TM expressed on Balb/3T3 cells. It has been shown that replacing the native GPI-anchor of DAF with different transmembrane domains does not affect its expression on the surface of the cell or its ability to regulate complement-mediated cell killing. To determine whether CD59 can also be anchored on the cell surface via a transmembrane moiety and still retain complement regulatory function, a recombinant transmembrane form of CD59 (CD59-TM) was generated. Balb/3T3 cells were transfected with CD59-TM and stable clones were tested for complement regulatory activity relative to cells transfected with native CD59 (CD59-GPI). To directly compare the complement inhibitory activity of the two molecules, clones were selected that expressed equivalent levels of CD59-GPI or CD59-TM by FACS analysis using an anti-CD59 polyclonal antibody (Fig 1A). Identical results were obtained using an anti-CD59 MoAb, MEM-43 (data not shown). Balb/3T3 cells expressing CD59-GPI or CD59-TM were tested for sensitivity to PIPLC cleavage. Approximately 80% of CD59-GPI expressed on the surface of these cells was effectively removed by PIPLC treatment (Fig 1B). Conversely, treatment of Balb/3T3 cells expressing CD59-TM with PIPLC did not affect surface expression of this transmembrane-anchored molecule (Fig 1C).

The Balb/3T3 clones expressing equivalent levels of CD59-GPI or CD59-TM were compared in a complement-mediated dye release assay. Cells were loaded with the cytoplasmatic dye, calcein AM, and membrane pore formation was measured as a function of dye release after exposure to human complement. The CD59-TM-expressing clone was equally effective in inhibiting membrane damage by human complement as compared with the CD59-GPI-expressing clone (Fig 2). For example, cells expressing these molecules were approximately threefold more effective in preventing complement-mediated membrane damage at 2.5 mg/mL C8/ C9 than cells transfected with vector alone. These results indicate that a recombinant transmembrane form of CD59 can be stably expressed on the surface of Balb/3T3 cells and that this molecule has comparable function to native GPI-anchored CD59.

Analysis of CD59-TM expressed on GPI-anchoring deficient mouse L cells. Mouse L cells are unable to express GPI-anchored proteins on the cell surface. Although the defect in these cells is not well understood, the mechanism appears to be related to the GPI-anchoring pathway. For instance, chimeric molecules consisting of the Q7 gene truncated at the GPI-anchor signal and the transmembrane domains of H-2 genes are successfully expressed on the surface of transfected L cells. To determine whether CD59-TM could be expressed on the surface of these cells and could protect them from human complement, L cells were transduced with the retrovirus LXSN containing CD59-TM. Cells transduced with LXSN containing CD59-GPI or LXSN alone were analyzed as controls. FACS analysis of transduced L cells selected as pools showed a wide range of CD59-TM expression, whereas CD59-GPI was not expressed (Fig 3A). In parallel, only L cells expressing CD59-TM showed protection against complement-mediated membrane damage as compared with cells transduced with the vector alone control (Fig 3B). These results show that the chimeric CD59-TM molecule can be successfully expressed
on the surface of a cell line that is unable to express GPI-anchored proteins and that the molecule functions to protect these cells from complement-mediated damage.

**CD59-TM expression in Epstein-Barr virus (EBV)-transformed PNH B cells.** It is well documented that PNH cells are deficient in the surface expression of GPI-anchored proteins including CD59. To investigate the potential of replacing CD59 on the surface of an EBV-transformed PNH B-cell line shown previously to be void of surface expressed GPI-anchored proteins, the cells were transduced with the LXSN retrovirus containing CD59-TM. After selection, cells were analyzed by Western blot analysis. The CD59-TM-transduced PNH B cells showed expression of a protein approximately 27 kD, which is identical to the predicted size of CD59 including the additional 81 amino acid transmembrane domain (Fig 4, lane 5). PBLs expressed a protein of 18 kD (lane 4) that corresponded to CD59 purified from human erythrocytes (lane 1). Nontransduced PNH B cells (lane 2) and PNH B cells transduced with vector alone (lane 3) showed no detectable levels of CD59.

FACS analysis of the transduced PNH B cells showed surface expression of CD59-TM (Fig 5A). By contrast, the nontransduced PNH B cells and the PNH B cells transduced with vector alone did not express CD59. These results indicate that CD59-TM can be successfully expressed on the surface of PNH B cells at a level comparable to that seen on the surface of PBLs. To confirm that CD59 expressed on the surface of the PNH B-cell transductants was transmembrane-bound, the CD59-TM—expressing PNH B cells as well as the PBLs were subjected to PIPLC treatment before FACS analysis. CD59 expression on the surface of PBLs was reduced approximately 85% after PIPLC cleavage (Fig 5B).

Conversely, the expression of CD59-TM on the surface of the PNH B cells was not affected by PIPLC treatment (Fig 5C).

**Functional analysis of CD59-TM—expressing PNH B cells.**
Absence of the terminal complement regulatory protein, CD59, on the surface of PNH cells is thought to be primarily responsible for the hemolytic anemia and thrombosis characteristic of this disease.\textsuperscript{23,24} To address the question of whether autologous complement regulation could be restored in PNH cells, the CD59-TM-expressing PNH B-cell line was assayed for protection against complement-mediated membrane damage. The CD59-TM--expressing PNH B cells showed resistance to complement damage (Fig 6A). For instance, at 32 ng/mL of human C8 input, the CD59-TM--expressing PNH cells were approximately twofold more effective at preventing complement-mediated membrane damage relative to the nontransduced PNH B cells. PNH B cells transduced with vector alone showed no protection.

**DISCUSSION**

The major clinical manifestations of PNH result from intravascular hemolysis and thrombosis.\textsuperscript{1,2,9} Data from several studies suggest that the complement-mediated pathology in these patients is primarily caused by the lack of the terminal complement regulatory protein, CD59, on the surface of erythrocytes and platelets.\textsuperscript{21-27} This deficiency is a result of the clonal expansion of a pluripotent hematopoietic stem cell containing a somatic mutation in an enzyme required for the initial step in GPI-anchor assembly (PIG-A).\textsuperscript{7,8} In this report, we have used a recombinant transmembrane form of CD59 to explore the possibility of correcting the increased complement sensitivity of PNH cells by stably expressing this molecule.

It has been hypothesized that a GPI-anchor confers increased lateral mobility to a protein on the membrane surface relative to a protein linked to the membrane via a transmembrane domain.\textsuperscript{30} Theoretically, this might allow more contact between a surface molecule and its ligand. However, in the present study, CD59-TM and CD59-GPI expressed at equivalent levels on the surface of stably transfected Balb/3T3...
cells showed equivalent levels of protection against complement-mediated membrane damage. These results parallel a recent study showing that a recombinant transmembrane form of DAF expressed in Chinese hamster ovary cells exhibits an equivalent level of complement regulatory activity when compared with the native GPI-anchored protein.

Thus, in both cases the replacement of the GPI-anchor of CD59 or DAF with a transmembrane domain does not appear to alter their capacity to regulate complement activity.

There are considerable data suggesting that CD59 is primarily responsible for the protection of erythrocytes and platelets from autologous complement. For instance, in vitro studies have shown that CD59-GPI incorporated into the membrane of PNH erythrocytes protects these cells from complement lysis. Additionally, CD59 has been shown to be critical for regulating C5b-9-mediated platelet procoagulant responses and PNH platelets lacking this molecule are an order of magnitude more sensitive to C5b-9 induced expression of prothrombinase activity when compared with the normal platelet. These data are supported by studies that show that patients with an inherited deficiency of DAF (Inab phenotype) but normal levels of CD59 exhibit no signs of hemolytic anemia or thrombosis. Conversely, a patient with an inherited deficiency of CD59 but normal levels of DAF presents with symptoms indistinguishable from PNH. Taken together, these data suggest that replacing CD59 alone would be sufficient to prevent complement-mediated hemolysis and thrombosis in PNH patients. Although erythrocytes that are deficient in the surface expression of DAF (Inab) have been shown to be more sensitive than the normal red blood cells to complement-mediated hemolysis initiated through activation of the classical pathway, this mode of activation is unlikely to be responsible for complement destruction of the PNH cells in vivo. Accordingly, the Inab erythrocyte is not sensitive to complement destruction mediated through the alternative complement pathway.

Considering the increased vulnerability to autologous complement attack, clonal expansion of the diseased PNH cell suggests that these cells have a survival advantage over the unaffected hematopoietic cells. The absence of GPI-anchored proteins on the surface of PNH cells suggests that one or more such proteins may contribute to a growth disadvantage for the unaffected cells. This has led to the hypothesis that PNH is not the primary lesion but rather a sequela to an underlying defect in hematopoiesis. This hypothesis is supported by the finding that PNH arises in up to 20% of patients with hypoplastic/aplastic anemia. Furthermore, in vitro studies have shown that cultures of PNH patient peripheral blood mononuclear cells and bone marrow cells show a reduction in both erythroid and myeloid progenitor cells. Although a GPI-anchored protein has not been identified as a target in bone marrow failure, possible candidates include LFA-3 and CD14, molecules involved in thymocyte adhesion and cell differentiation, respectively. Additionally, antigens recognized by natural killer (NK) cells appear to be GPI-anchored. This hypothesis might also imply that somatic mutations in PIG-A occur in the normal population but are only propagated when the selective pressure of bone marrow failure is present. Indeed, the characterization of multiple mutations in the PIG-A gene between patients suggests a random mutational event. These data correlate with studies showing that more than one population of PNH erythrocytes is often found in patients and that differences between clones result from distinct mutations in the PIG-A gene.

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marrow transplantation in the future if the ability to efficiently transduce stem cells is achieved.

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