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.

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. The hemolytic anemia associated with PNH can be attributed to increased C5b-9 deposition on erythrocytes. 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. 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. DAF regulates complement activation by inhibiting C3 convertase, whereas CD59 inhibits the assembly of C5b-9 by interacting with C8 and C9.

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 (Inab phenotype), but expressing normal levels of CD59, show no clinical signs of hemolytic disease or thrombosis. However, a patient genetically deficient in the expression of functional CD59 but expressing normal levels of DAF exhibits symptoms indistinguishable from PNH. In vitro studies have supported these results because isolated erythrocytes with the Inab phenotype resist complement-mediated lysis initiated through the alternative pathway. Additionally, purified CD59 incorporated into the membrane of the type III PNH erythrocyte protects these cells from complement damage. 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.

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

**MATERIALS AND METHODS**

*cDNA constructs.* A transmembrane form of HuCD59 (CD59-TM) was constructed by replacing the putative GPI-anchor signal of CD59 with the carboxy-terminal domain of the MCP. CD59 was truncated at amino acid 77 of the mature protein (CD59-GPI) through polymerase chain reaction (PCR) mutagenesis and an Ssp I site was introduced for subcloning. The carboxy-terminus of MCP was PCR-amplified from HeLa cell reverse-transcribed mRNA using the following primers: 5′-CGCGAAGCTACTAACAAGCCTCCAG-3′ and 5′-CCGGCTATCATGAGGCTCTGCTTGTC-3′. 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. An endogenous Ssp I site found at the 5′ end of the fragment was used to ligate this domain to the 3′ end of CD59-GPI, 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. CD59-GPI and CD59-TM were further subcloned as BamHI fragments into the retroviral vector pLXSN.

**Amphotropic virus production.** Amphotropic virus was produced through an intermediate ecotropic packaging cell line as previously described. 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 pCDNA 3 containing CD59-GPI, CD59-TM, or pCDNA 3 alone using the calcium phosphate method as previously described. Cells were selected in D10 medium containing 500 mg/mL of G418 and colonies were isolated using cloning cylinders. Mouse L cells (ATCC) and the PNH B-cell line HL/677 were transduced with amphotropic virus stocks by adding 0.5 mL of virus to 5 × 10⁶ cells in growth media containing 8 mg/mL polybrene. After an overnight incubation, virus was removed and selection media was added (500 mg/mL G418). After 14 days on selection, L cells were pooled and analyzed by fluorescence-activated cell sorting (FACS). B cells were selected for 6 weeks before analysis.

**CD59 surface expression.** Transfected or transduced cells were screened for surface expression of CD59-GPI or CD59-TM by indirect immunofluorescence using polyclonal and monoclonal antibody (MoAb) preparations. Human CD59 polyclonal #349 was provided by Dr Peter Sims (Blood Research Institute, Milwaukee, WI). The CD59 MoAb, MEM-43, was purchased from Biodesign International (Kennebunkport, ME). Cell surface indirect immunofluorescence was performed on 2 × 10⁶ cells with 50 mg/mL of the polyclonal 349 or 20 mg/mL of MoAb MEM-43 in 1× phosphate-buffered saline (PBS) with 2% fetal bovine serum. Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG or goat antimouse IgG antibodies were used as a secondary antibody (Zymed Laboratories, South San Francisco, CA). Immunofluorescence was measured by FACS using a Becton Dickinson FACSort (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). In experiments to determine the sensitivity of CD59-TM to phosphatidylinositol-specific phospholipase C (PIPLC), Balb/3T3-transfected cells or transduced B cells were treated with PIPLC (Boehringer Mannheim, Indianapolis, IN) at 1 U/mL for 1 hour at 37°C before staining and subsequent FACS analysis.

**Immunoprecipitation and Western blot analysis.** Transduced or nontransduced B cells (5 × 10⁶) or peripheral blood lymphocytes (PBLs) were incubated in 1 mL of PBS lysis buffer (0.5% deoxycholate, 1% nonidet NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.23 U Aprotinin) for 15 minutes on ice and the nuclei were removed by centrifugation at 13,000 g for 15 minutes. The supernatants were incubated overnight at 4°C with 10 mg/mL of anti-CD59 polyclonal Ab (ALP-3; generated in-house) followed by the addition of 100 mL of agarse bead-coupled goat antirabbit Ab (Sigma Chemical Co, St Louis, MO) for 2 hours. The beads were washed twice in PBS lysis buffer before resuspending in 50 mL of nonreducing sample buffer. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and transferred to nitrocellulose for Western blot analysis, as described elsewhere. ALP-3 was used as the primary Ab and the blot was developed with an alkaline phosphate-conjugated goat antirabbit Ab (Zymed Laboratories) and Western Blue-stabilized substrate, as suggested by the manufacturer (Promega Corp, Madison, WI).

**Complement-mediated dye release assay.** Complement-mediated membrane damage of Balb/3T3 cells, L cells, and B cells transfected or transduced with CD59-GPI, CD59-TM, or vector alone was assessed by measuring the release 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 Hanks’ 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 Cs5b, 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 nonspecific 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 × 10⁶) were washed three times with GVBS++ (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 mg/mL 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 was 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.31 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 cytoplasmic 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 C8/9 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.32 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.33 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 transfected with the vector alone control (Fig 3B). These results show that the chimeric CD59-TM molecule can be successfully expressed
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Fig 2. Complement regulatory activity of CD59-TM relative to CD59-GPI. Stably transfectants expressing equivalent levels of CD59-TM or CD59-GP1 by FACS analysis (Fig 1) were subjected to a complement-mediated dye release assay. Complement-mediated membrane damage was measured as the percentage of dye release after the addition of various concentrations of complement (see the Materials and Methods) relative to the total amount of dye taken up by the cells. Curves represent dye release from cells expressing CD59-TM (TM), CD59-GP1 (GPI), or cells transfected with vector alone (Control). Data shown are means of duplicate determinations from one experiment, one of four so performed.

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


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).

Fig 3. Expression and functional analysis of recombinant CD59-TM in GPI-anchor deficient mouse L cells. Mouse L cells transduced with CD59-TM, CD59-GP1, or vector alone were selected with G418. The transfected pool was stained with polyclonal anti-CD59 Ab and analyzed by FACS. A. Surface expression of cells transduced with CD59-TM (TM) or CD59-GP1 (GPI) relative to a clone transfected with vector alone (Control) are shown. The transfected cells were subjected to a complement-mediated dye release assay as described in Fig 2 (B). Curves represent dye release from cells transfected with CD59-TM (TM), CD59-GP1 (GPI), or vector alone (Control). Data shown are means of duplicate determinations from one experiment, one of three so performed.
Fig 4. Western blot analysis of recombinant CD59-TM expression in PNH B cells. A PNH B-cell line lacking GPI-anchored proteins was transduced with CD59-TM or vector alone and cells were selected as a pool in G418. Transduced cells or PBLs were lysed and immunoprecipitated using a polyclonal anti-CD59 Ab. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and reacted with anti-CD59 Ab. Lane 1, control CD59 purified from human erythrocytes; lane 2, nontransduced PNH cell immunoprecipitate; lane 3, immunoprecipitate from PNH cells transduced with vector alone; lane 4, immunoprecipitate from PBLs; lane 5, CD59-TM transduced PNH B-cell immunoprecipitate. Protein size in kilodaltons is indicated on the left of the figure. This figure represents one of two experiments so performed.

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.\(^{23,24}\) 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 non-transduced 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.\(^{1,2,9}\) 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.\(^{21-27}\) 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).\(^{7,8}\) 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.\(^{10}\) Theoretically, this might allow more contact between a surface molecule and its ligand. However, in the present study, CD59-TM and CD59-GP1 expressed at equivalent levels on the surface of stably transfected Balb/3T3...
hemolysis initiated through activation of the classical path-
mediated hemolysis and thrombosis in PNH patients. Al-
though erythrocytes that are deficient in the surface expres-
tion of PIG-A23,24 Taken together, these data suggest that replacing
DAF presents with symptoms indistiguishable from
with an inherited deficiency of CD59 but normal levels of
hemolytic anemia or Conversely, a patient
the normal platelet.' These data are supported by studies that
expression of prothrombinase activity when compared with
complement lysi~. Additionally, CD59 has been shown
membrane of PNH erythrocytes protects these cells from
platelets from autologous complement. For instance, in vitro
studies have shown that CD59-GP1 incorporated into the
expression of a recessive phenotype resulting from a somatic
mutation and more than likely explains the inability to find
other affected genes in the GPI-anchoring path~ay.29
There are considerable data suggesting that CD59 is pri-
marily 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.26,27 Additionally, CD59 has been shown
to be critical for regulating C5b-9-mediated platelet procoag-
ulant responses22 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.2 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.21,22 Conversely, a patient
with an inherited deficiency of CD59 but normal levels of
DAF presents with symptoms indistinguishable from
PNH.23,24 Taken together, these data suggest that replacing
CD59 alone would be sufficient to prevent complement-
mediated hemolysis and thrombosis in PNH patients. Al-
though erythrocytes that are deficient in the surface expres-
sion 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 path-
way, this mode of activation is unlikely to be responsible
for complement destruction of the PNH cells in vivo. Ac-
cordingly, the Inab erythrocyte is not sensitive to comple-
ment destruction mediated through the alternative comple-
ment pathway.21,25
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-anch-
ored proteins on the surface of PNH cells suggests that
one or more such proteins may contribute to a growth disad-
vantage for the unaffected cells. This has led to the hypothe-
sis that PNH is not the primary lesion but rather a sequela
to an underlying defect in hematopoiesis.1 This hypothesis
is supported by the finding that PNH arises in up to 20% of
patients with hypoplastic/aplastic anemia.41,44 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.45 Although a GPI-anchored protein has not been identi-
fied as a target in bone marrow failure, possible candidates
include LFA-3 and CD14, molecules involved in thymocyte
adhesion and cell differentiation, respectively. Additionally,
tagens recognized by natural killer (NK) cells appear to
be GPI-anchored.46 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 sug-
uggests a random mutational event.47,48 These data correlate with
studies showing that more than one population of PNH eryth-
rocytes is often found in patients and that differences be-
tween clones result from distinct mutations in the PIG-A
gene.47,48 (Hillmen et al, manuscript in preparation). Localization
of the PIG-A gene to the X chromosome allows for the
expression of a recessive phenotype resulting from a somatic
mutation and more than likely explains the inability to find
other affected genes in the GPI-anchoring pathway.7
Although in vivo gene therapy has met with limited suc-
cess, it is intriguing to consider CD59-TM as a candidate
for such a therapy. If somatic mutations in the PIG-A gene
are responsible for the primary lesion in PNH, then PIG-
A will be the obvious molecule for potential gene therapy
treatment. However, considering the evidence above sug-
gesting that the primary lesion in PNH is bone marrow fail-
ure and that the appearance of PNH cells is a sequela of this
disease, replacement of the PIG-A gene may again target
these cells for destruction. CD59, on the other hand, has
been shown to be critical in protecting platelets and erythro-
ocytes from autologous complement-mediated activation and/or
lysis. Restoring CD59 in a hematopoietic stem cell with a
transmembrane form of the molecule may prevent lysis of
PNH erythrocytes and platelet activation without replacing
the signal that is responsible for cell destruction. Bone mar-
row transplants have been successfully used to eradicate
PNH in isolated cases,49,51 but in most instances the lack of
severity of the disease does not warrant the risks associated
with the procedure. The introduction of CD59-TM through
retroviral gene therapy could provide an alternative to bone

cells showed equivalent levels of protection against comple-
ment-mediated membrane damage. These results parallel a
recent study showing that a recombinant transmembrane
form of DAF expressed in Chinese hamster ovary cells ex-
hibits an equivalent level of complement regulatory activity
when compared with the native GPI-anchored protein.51
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.
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than the normal red blood cells to complement-mediated hemolysis initiated through activation of the classical path-
marrow transplantation in the future if the ability to efficiently transduce stem cells is achieved.

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