86 Paroxysmal Nocturnal Hemoglobinuria

Bruno Rotoli, Khedoudja Nafa, and Antonio M. Risitano

SUMMARY

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic disorder arising from a mutation in the X chromosome-linked *PIG-A* gene. The mutated cell is unable to build the GPI anchor, which is needed to bind a number of molecules on the outer cell surface. Some GPI-linked molecules are responsible for cell defense against activated complement; this is why PNH red cells are hypersensitive to complement attack and are continuously destroyed in the circulation, with paroxysmal exacerbations. However, many other defects are present on PNH cell membrane, involving red cells, granulocytes, monocytes, and platelets, leading to tendency to infection and thrombosis. This review highlights the molecular pathophysiology of the disease and analyses the mechanisms responsible for the expansion of a hematopoietic clone, which is paradoxically less vital as compared with normal cells.

Key Words: Aplastic anemia; bone marrow failure; CD55/DAF; CD59/MIRL; clonal hematopoiesis; complement-mediated lysis; escape theory; GPI anchor; GPI-linked molecules; intravascular hemolysis; pancytopenia; paroxysmal nocturnal hemoglobinuria; *PIG-A* gene; T-cell clonality; thrombophilia.

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a complex hematological disorder probably first described three centuries ago, and regarded as a mystery until the 1980s, when most of its pathophysiology was elucidated, followed by the 1990s, when the underlying molecular defect was finally unraveled. The original denomination, PNH, stresses only one component of the disease (i.e., a hyperhemolytic state). A more complete updated definition could read as follows: an acquired blood disorder characterized by the expansion of one or a few hematopoietic cell clones carrying a mutation in the X-linked phosphatidylinositol glycan class A *(PIG-A)* gene, which renders the cells unable to produce the glycosyl-phosphatidylinositol (GPI) anchor, against a background of a reduced bone marrow activity. Some landmarks in the history of understanding this disorder are listed in Table 86-1. Because of its complex pathophysiology, the disease has been variously classified among hemolytic anemias, myelodysplasia, myeloproliferative disorders or bone marrow failure syndromes; indeed, PNH has some features of each of these. This chapter reviews advances on genetic and pathophysiologic issues of PNH, including a summary of clinical, diagnostic, and therapeutic aspects.

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GENETIC BASIS

Although this disease has a well-recognized genetic cause, it is neither inherited nor transmitted to the progeny. Indeed, it is a clonal disorder arising from a somatic mutation in the *PIG-A* gene of an early hematopoietic progenitor.

THE *PIG-A* GENE AND THE GPI ANCHOR *PIG-A* is a housekeeping gene located on the short arm of the X chromosome (Xp22.1), the cDNA of which was first isolated in 1993 through expression cloning and complementation of GPI-deficient cell lines. The organization of the genomic gene was established by Bessler in 1994 (Fig. 86-1). This gene encodes for a protein involved in the synthesis of the GPI anchor, a molecule used by cells to bind a number of proteins on their own outer surface without a transmembrane portion. The functional implications of the GPI anchoring of proteins are not completely understood; they include ease of assemblage and shedding, lateral mobility, capping, involvement in endo-, exo-, and potocytosis (a clathrin-independent form of endocytosis and recycling). There are examples of proteins existing in both transmembrane and GPI-linked configuration, as a result of various molecular mechanisms. The adhesion molecule leukocyte function associated (LFA)-3 (CD58) is encoded by a gene that may undergo alternative splicing producing two different polypeptides, one of which provides the site for GPI-anchor attachment. Instead, two alternative isoforms of the Fc-γ receptor III (CD16) result from two different genes expressed in a tissue-specific fashion (GPI-linked in neutrophils and transmembrane in natural killer [NK] cells). The strongest evidence that this type of membrane anchoring is important in cell biology is its high conservation among eukaryotic cells; indeed, it is found even in yeast and trypanosome. The protein encoded by *PIG-A*, in combination with at least two other proteins, is essential in the first step of the complex GPI-anchor biosynthesis pathway, i.e., in the transfer of *N*-acetyl glucosamine to phosphatidyl inositol (Fig. 86-2). By experimental mutagenesis, GPI-deficient cell lines showing defects in any of the various metabolic steps have been produced; however, the study of PNH has shown that the same early step is impaired in all patients, and all patients have a mutation in the *PIG-A* gene. The explanation for this finding is that among the various genes involved in the GPI-anchor synthesis, *PIG-A* may be the only one that is X-linked. As a consequence, a single mutation in that gene will produce an abnormal cell in either sex: males

Table 86-1
Landmarks in the History of PNH

Year	*Scientist*	*Issue*
1678	Schmidt	First clinical report
1882	Strübing	First detailed description
1911	Marchiafava	Recognition as disease entity
1937	Ham; Dacie	Specific diagnostic test
1956	De Sandre	PNH erythrocytes are AchE deficient
1965	Lewis and Dacie	PNH granulocytes are NAP-deficient
1966	Rosse and Dacie	Two types of PNH erythrocytes
1967	Lewis and Dacie	Relation with aplastic anemia
1970	Oni and Luzzatto	Evidence of clonal origin
1983	Nicholson-Weller	PNH cells are DAF-deficient
1984	Rotoli and Luzzatto	In vitro evidence of clonal origin
1986	Medof	Relation to GPI-anchoring system
1989	Rotoli and Luzzatto	The survival advantage hypothesis
1990	Van der Schoot	Cytometry of GPI-deficient cells
1992, 1993	Ueda; Hillmen	PNH lymphoblastoid cell lines
1993	Kinoshita	Cloning of the *PIG-A* gene
1994	Bessler and Luzzatto	*PIG-A* gene organization and mutations
1999	Araten	PNH cells in normal individuals
2001	Bessler	Mouse models
2001, 2002	Karadimitris; Risitano	Clonal T-cell expansion in PNH patients

AchE, acetylcholinesterase; DAF, decay accelerating factor (CD55); GPI, glycosyl-phosphatidylinositol; NAP, neutrophil alkaline phosphatase; PIG-A, phosphatidylinositol glycan class A; PNH, paroxysmal nocturnal hemoglobinuria.

have only one allele and females have only one functional allele (as result of X-chromosome inactivation). Although females have two *PIG-A* alleles, only half of the mutation will occur in the functional X; thus, the risk of having the disease is the same in both genders. Because a defect causing a metabolic block is generally recessive, it is unlikely (although not impossible) that a double mutation targeting both alleles of an autosomal gene in the same cell may occur in vivo.

***PIG-A* MUTATIONS IN PNH** Practically all types of mutations have been found in the *PIG-A* gene of PNH cells: small deletions or insertions producing frameshift, nucleotide substitutions resulting in stop codon, missense mutations causing amino acids substitutions or new sites for alternative splicing, large deletions. Most of the mutations occur in exon 2, probably because it is the largest; however, no particular clustering of mutations has emerged, and almost all mutations are unique to each patient (private mutations) (*see* Fig. 86-1).

Comparing the type of mutations found in PNH with those found in glucose-6-phosphate dehydrogenase deficiency, another (this time inherited) X-linked disorder of a housekeeping gene, shows a clear discrepancy. In PNH, a vast majority of mutations have extremely severe functional consequences, (i.e., production of truncated proteins), whereas missense mutations are rare; in glucose-6-phosphate dehydrogenase deficiency almost all mutations are missense, leading to single amino acid substitutions with conserved (although possibly altered) function. Two considerations may be relevant to this discrepancy: (1) In hereditary disorders, a complete absence of the product of a housekeeping gene is probably lethal; thus, only mutations with residual activity are seen. By contrast, if a mutation is somatically acquired, it will not affect organ development and, once occurred, it will affect the genetic make-up of only a small fraction of somatic cells, which may survive although with some functional abnormality. (2) If missense mutations in PNH are rare, this means that for a clonal expansion to take place, the gene must be seriously damaged. Indeed, the rare missense mutations found in PNH patients are associated with a marked reduction of GPI-linked proteins, indicating that they affected mRNA stability or protein function, or both.

MOLECULAR PATHOPHYSIOLOGY

The *PIG-A* mutation responsible for PNH arises in a multipotent hematopoietic stem cell, which is capable of differentiating along several lineages; maturation along erythroid, myeloid, and megakaryocytic lineages is almost always preserved, whereas lymphoid differentiation (along B- and/or T lineages) is observed in a minority of patients. As a consequence of the damage of the GPI-anchor synthesis pathway, all the affected cells in any state of maturation are deficient in all the GPI-linked molecules, and deficiency is often complete. More than 20 different proteins sharing the GPI-anchoring system have been described on the surface of human blood cells (Fig. 86-3). The understanding of their functional significance is limited; in many cases, the study of PNH patients or cells was an important source of information.

HEMOLYSIS IN PNH PATIENTS Intravascular hemolysis is the most characteristic clinical feature of PNH; it is because of increased susceptibility of PNH erythrocytes to complement-mediated lysis. This abnormality is caused by the absence on red cell membrane of two complement-regulatory proteins, CD59/MIRL (membrane inhibitor of reactive lysis) and CD55/DAF (decay accelerating factor), which are both GPI-linked. This membrane abnormality renders PNH erythrocytes extremely sensitive to complement activation, which translates in vivo into a hemolytic state and in vitro into red cell destruction in acified serum (Ham test). A low level of complement activation is probably a constant physiologic phenomenon, which is greatly amplified during inflammatory or infectious diseases; thus, PNH erythrocytes are chronically lysed with paroxystic exacerbations. It is unclear why exacerba-

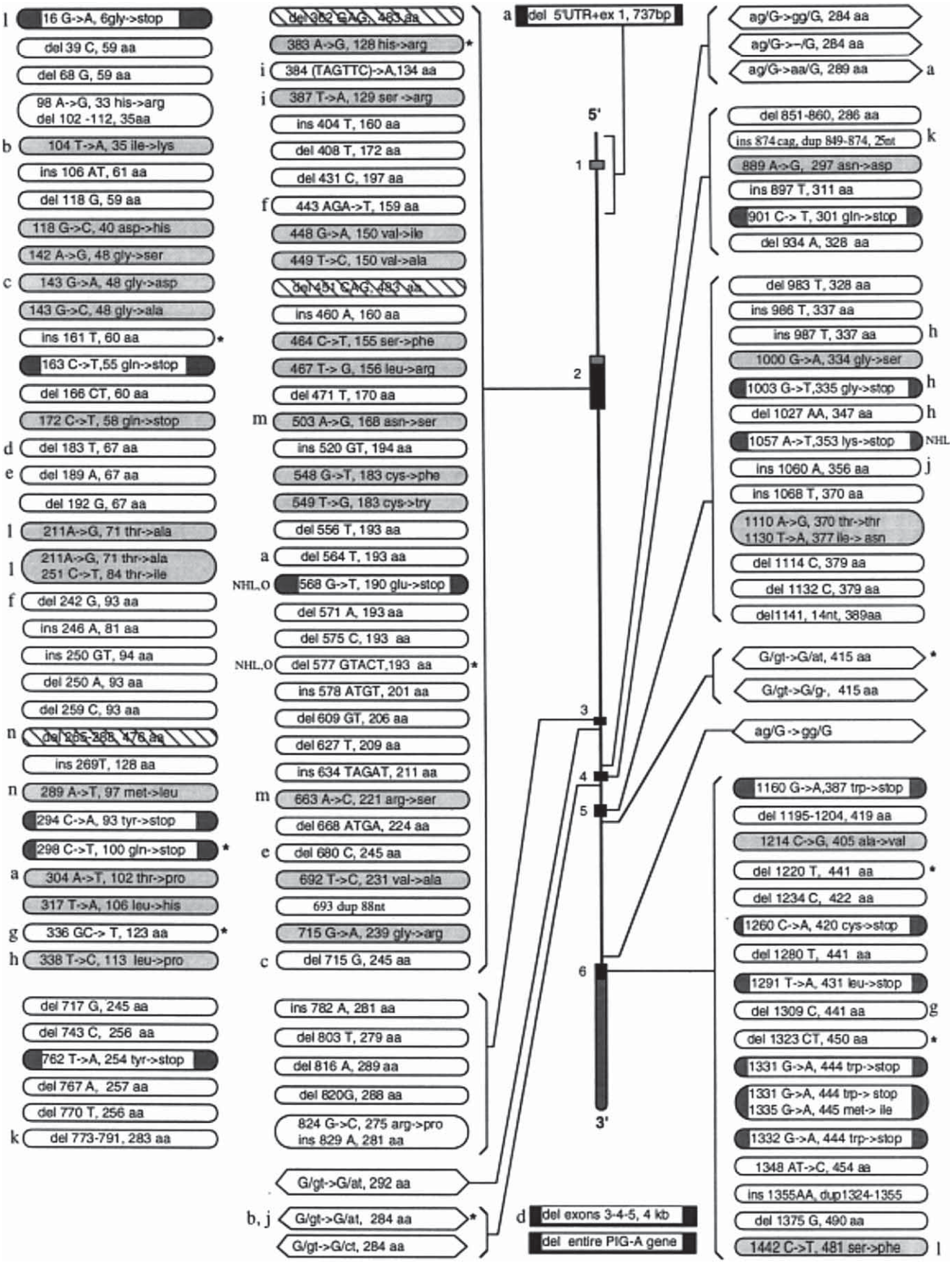

Figure 86-1 Phosphatidylinositol glycan class A gene structure and mutations. The genomic gene spans approx 17 kb. Exons (thick blocks, numbered) and introns (thin lines) are represented in the central bar; untranslated exon regions are in gray. Three mRNA transcripts arising from alternative splicing were found by Bessler et al., (1994) who also described an unproductive pseudogene mapped to 12q21. Mutations found to

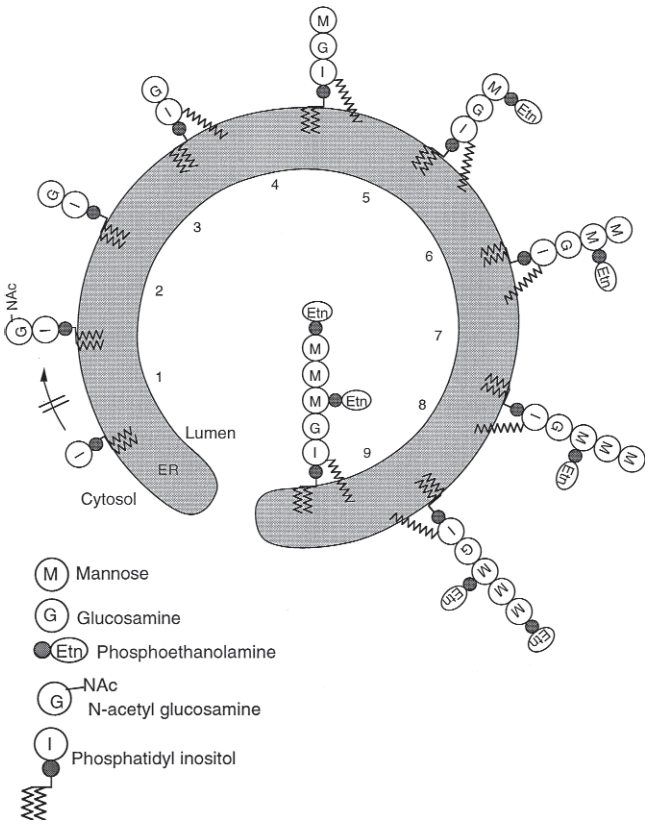

Figure 86-2 Glycosyl-phosphatidylinositol-anchor biosynthesis. The anchor synthesis is a multistep (1–9) process that occurs in the endoplasmic reticulum (ER), starting on the cytoplasmic side, then flipping into the luminal side. The anchor is transferred *en bloc* on the C-terminal of the protein, which is cleaved of a short sequence of 20–30 amino acids led by a signal sequence called ω. Although GPI-deficient cell lines obtained by experimental mutagenesis have shown defects in any step of the synthesis, all paroxysmal nocturnal hemoglobinuria patients studied so far are defective in the first step (⊣⊢).

tions occur mainly during the night. In a functional hierarchy, CD59 plays a major role, as discovered by studying rare inherited conditions in which a single molecule is lacking. A CD59-deficient patient had a PNH-like picture, whereas subjects deficient of CD55 on red cell membrane (those carrying the rare Inab blood group phenotype) show no hyperhemolysis. Although CD59 and CD55 are deficient also on leukocytes, there is no evidence of reduced leukocyte life-span in PNH patients, probably because nucleated cells have an additional transmembrane (i.e., non-GPI-linked) molecule (CD46) that protects them from complement activation. Sensitivity to complement is the best known, but not the only defect, of PNH cells. Table 86-2 lists other molecules that are deficient and their specific function, when known.

HEMATOPOIESIS IN PNH PATIENTS A wide spectrum of clinical picture emerges when several PNH patients are compared with each other, ranging from almost asymptomatic individuals to severely ill subjects. Several mechanisms are responsible for such phenotypic heterogeneity:

1. The coexistence of a variable percentage of normal cells, which are progeny of residual GPI+ stem cells.
2. A selective destruction of particles succumbing to the activated complement attack (this is particularly true for PNH red cells after a paroxysm of hemolysis).
3. The type of molecular lesion in the *PIG-A* gene, leading to PNH cells with a variable sensitivity to complement-mediated lysis (PNH II and PNH III populations).
4. The degree of commitment of the stem cell having the *PIG-A* mutation; if the mutation occurs in a totipotent stem cell, even lymphocytes will carry the PNH defect, whereas a mutation in a multipotent myeloid cell will not produce PNH lymphocytes.

In theory, a mutation in a more committed progenitor could generate a monolineage PNH; however, patients with a purely myeloid *PIG-A*-deficient clone, giving rise to only granulocytes and/or platelets may remain undetected, as the sparing of the erythroid lineage implies lack of the typical symptoms suggesting PNH.

PNH hematopoiesis is certainly clonal, but clonal does not necessarily mean monoclonal. Initial observations that more than one abnormal cell population may be present in a single PNH patient were first inferred by functional studies based on different sensitivity to complement-mediated lysis and then by flow cytometry in the early 1990s. The final confirmation of such an unusual pattern came from DNA analysis, which documented the simultaneous presence of two or even more different *PIG-A* mutations in several PNH patients, each originating a distinct multilineage PNH clone. A mechanism that may explain the expansion of more clones carrying the same functional defect but arising from different mutations is selection. A theory based on this mechanism was developed for PNH in 1989 and termed the "relative advantage," later the "escape" hypothesis; it is supported by several pieces of circumstantial evidence, but still requires a direct confirmation. According to this theory, a mutation in the *PIG-A* gene might be a fairly common phenomenon, which has no biological consequences, because the mutated cell has no chance of expanding in the presence of a vast majority of normal cells. However, if normal hematopoiesis is inhibited by a primary cause (as in idiopathic aplastic anemia [AA]), and if the killing mechanism is directly or indirectly mediated by one or more proteins that are GPI-linked on the surface of progenitor cells, *PIG-A* mutated cells survive, undergoing clonal expansion. Because only cells severely GPI-deficient are able to survive, in PNH serious molecular lesions (i.e., frameshift, stop codon, and deletion) prevail over single amino acid substitutions. Several observations indirectly support this theory; in patients with a B-cell lymphoproliferative disorder treated by Campath-1H (a monoclonal antibody that kills lymphocytes targeting the GPI-linked protein CD52), severely GPI-deficient T cells emerged; they were cloned and showed mutations in the *PIG-A* gene. When a banked blood specimen that had been drawn before monoclonal antibody treatment was searched for the *PIG-A* deficient clone, a few cells carrying the same molecular defect were detected. On Campath-1H discontinuation, the GPI-deficient T cells gradually disappeared. Thus, this model elucidates most features of the PNH pathogenesis: the preexistence of *PIG-A*

Figure 86-1 ***(Continued)*** date are displayed. ⬭: small deletion or insertion and resulting in frameshift and truncated protein; ⬭: nonsense mutation; ⟷: mutation causing altered splicing; ▭: vast deletion; ▨: deletion, in frame; ▬: missense mutation. *Mutations found in more than one patient. A small letter outside the box identifies multiple mutations found in a single patient. Only one polymorphism has been described (55C→T, 19 arg→trp).

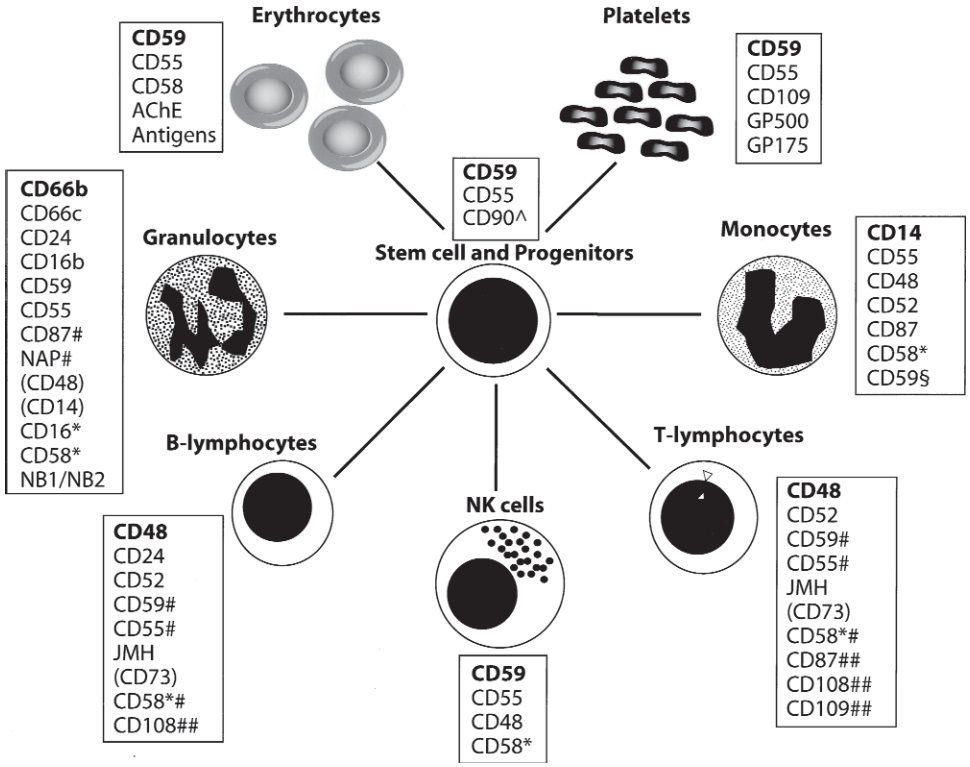

Figure 86-3 GPI-linked molecules on blood cell surface. For each cell type, the molecules are listed in order of sensitivity for detecting deficient cells (in bold, highest sensitivity; molecules in parenthesis, weak expression; *, also transmembrane; #, increased on cell activation; ##, expressed only on cell activation. ^, approx 25% of early hematopoietic progenitors express CD90, which is the analog of murine Thy-1. §, in our hands, CD59 was often present on PNH monocytes. A few molecules have been omitted that need better knowledge of their lineage-restricted expression (ULBPs, PRV-1, PrP^c, TRAIL-R3).

mutated cells, their expansion under a selective pressure negatively acting on normal cells by a mechanism to which the mutated cells are resistant (for the absence of CD52), and the gradual disappearance of the mutated clone once the selective mechanism has been removed. The existence of a few (10–50 cells per million) PNH cells in peripheral blood of healthy individuals has been documented by analyzing cytometrically a large number of granulocytes. A nested PCR technique confirmed the clonality of these cells by identifying the specific *PIG-A* mutation. The absence of an intrinsic genomic instability in PNH patients has been shown by comparison with another bone marrow failure disorder, Fanconi anemia (FA). The measurement of *PIG-A* spontaneous mutation rate in B-lymphoblastoid cell lines derived from PNH or FA patients showed a normal rate of mutation in PNH patients, contrasting with a markedly elevated rate in FA patients. Thus, the development of PNH cannot be attributed to increased risk of *PIG-A* mutations, but only to the expansion of pre-existing rare mutant clones. The finding that FA patients, although having both more *PIG-A* mutated stem cells and reduced marrow cellularity, do not develop PNH, strongly supports the escape theory: because bone marrow failure in FA is owing to a mechanism that does not involve GPI-linked molecules, there is no growth advantage for the mutated cells.

Murine Models of PNH To explore experimentally the growth of PNH hematopoietic stem cells, murine models have been generated. A complete knockout animal could not be produced, demonstrating that *pig-a* is necessary for embryogenesis. When the mouse *pig-a* gene was inactivated in embryonic stem (ES) cells with the conventional knockout gene targeting technique, in vitro studies demonstrated that *pig-a* deficient ES cells were able to differentiate into mature cells of various hematopoietic lineages, showing that *pig-a* is not necessary for differentiation and maturation of hematopoietic progenitors. When tested in vivo, with chimeras obtained by inserting *pig-a* knocked out ES cells during early embryonic development, in the few surviving chimeras the proportion of GPI-deficient blood cells was low at birth and decreased with aging, proving that there is no absolute growth advantage for PNH cells as compared with normal cells coexisting in the same organism. Mice showing in vivo expansion of *pig-a* mutated hematopoiesis, thus better mimicking the human PNH disease, have been obtained with a more sophisticated approach. A conditional inactivation of the murine *pig-a* gene was implemented using the Cre recombinase and its specific recombination sites, loxP. When the Cre/loxP system was targeted to the hematopoietic stem cells or the erythroid/megakaryocytic lineage using tissue-specific c-FES and GATA-1 regulatory sequences, respectively, the generation of mice having almost 100% of red cells with the PNH phenotype was obtained. Such mice are under investigation to answer open questions in PNH pathophysiology.

In Vitro Decreased Susceptibility of PNH Cells to Immune Effector Mechanisms Several contradictory data have been produced on a putative differential sensitivity of normal and PNH hematopoiesis to inhibitory stimuli. Susceptibility to apoptosis has been reported increased, normal, or decreased in different models; it has been shown that human cell lines carrying the PIG-A mutation are less susceptible to NK-mediated killing compared with their normal counterpart, a phenomenon that was reverted by transfection with a *PIG-A* cDNA. In a more sophisticated model, GPI-deficient

Table 86-2
GPI-Linked Proteins on Blood Cells and Their Function

Antigen	*Alternative denomination*	*Function*
CD14	LPS-LPB-r	Monocyte adhesion and activation
CD16	FcγR-IIIb	Low affinity receptor for IgG
CD24		Ca^{2+} flux, triggers H_2O_2 production by polymorphonuclear leukocytes
CD48	BLAST-1	Counter-receptor for CD2
CD52	Target of Campath-1	Unknown
CD55	DAF	Accelerates C3 and C5 convertase decay, Cromer antigen
CD58	LFA-3	Counter-receptor for CD2
CD59	MIRL	Binds C8/C9, thus inhibiting MAC assembly
CD66b	CGM6 (CEA family member)	Granulocyte adhesion/activation
CD66c	NCA	Granulocyte adhesion/activation
CD73	5′-NT	Purine/pyrimidine ribo/deoxyribonucleoside phosphorylation
CD87	uPAR	Urokinase receptor
CD90	Thy-1 analog	Adhesion
CD108		Unknown
CD109	Platelet Gove/b alloantigen	Unknown
CD157	BST-1	Enzyme; adhesion/signaling (CD38 family)
ACHE	Red cell acetylcholinesterase	Enzyme, unknown substrate on RBC
NAP	Neutrophil alkaline phosphatase	Enzyme, granulocyte function
PrP^c	Cellular prion protein	Unknown
TRAIL R-3	Trail receptor III; DcR-1	Decoy receptor for TRAIL
ULBPs	UL-16 binding proteins	Activating ligand for NKG2D/DAP10 receptor
PRV-1	Polycythemia rubra vera 1	Unknown
NB1/NB2		Neutrophil antigen
JMH	John Milton Hagen antigen	Red cell antigen
Holley Gregory		Red cell antigen
Dombrock		Red cell antigen
YT		Red cell antigen
Platelet GP500		Platelet antigen
Platelet GP175		Platelet antigen

LFA, leukocyte function associated; uPAR, urokinase plasminogen activator.

cells showed impairment in inducing primary and secondary stimulation of both antigen-specific and alloreactive T cells, providing experimental support for the hypothesis that the PNH clone could inefficiently interact with the immune system. Higher apoptotic rate of GPI+ progenitor cells compared to GPI-deficient progenitors from the same PNH patient has been also documented. A new family of molecules has been identified: UL16 binding proteins (ULBPs). They are GPI-linked proteins belonging to the major histocompatibility complex class I superfamily, expressed in several tissues including immature and mature blood cells. They bind the activating receptor NKG2D/DAP10, which is expressed on NK and CD8+ cells, thus generating a dominant and potent stimulus for innate and adaptive immunity; the lack of ULBPs on PNH cells may explain the differential susceptibility to apoptosis of GPI+ and GPI– cells, providing a rationale for escaping from immune attacks.

However, the exact mechanisms causing the escape are elusive. They may include the absence of one or more GPI-linked molecules directly targeted by T lymphocytes or NK cells and leading to apoptosis of GPI+ cells; nonspecific structural changes of the raft structure on the membrane outer surface; and impaired sensitivity to common effector mechanisms involving GPI-linked molecules.

Evidence of an Immune-Mediated Disorder in PNH Patients An immune attack against normal hematopoietic progenitors may explain marrow failure in PNH and the close boundary with idiopathic AA, an immune-mediated disorder. Antigen-driven immune responses are characterized by in vivo oligoclonal expansion of specific T-cell subsets, which become dominant over the residual normal repertoire. T-cell receptor analysis represents a powerful tool for detection of clonality. In PNH, oligoclonality of the T-cell receptor repertoire has been documented by some groups through molecular analysis of the β-chain complementary determining region 3, by both size-distribution analysis (spectratyping or immunoscope, Fig. 86-4) and direct sequencing of dominant clonotypes. In some instances, a PNH clone was associated with the presence of extremely expanded T-cell subsets, resembling large granular lymphocyte leukemia. These observations are consistent with a T-cell response involving immunodominant cytotoxic T-lymphocyte clones, a mechanism already advocated for idiopathic AA; it may explain marrow failure in PNH, even if the putative triggering antigens have not been identified.

If the escape theory is confirmed, several implications can be drawn:

1. PNH patients always have two concomitant defects: one (or a few) early hematopoietic progenitors carrying a *PIG-A* mutation and a bone marrow failure syndrome, the mechanism of which is permissive for the expansion of GPI-deficient cells. This explains the frequent association of

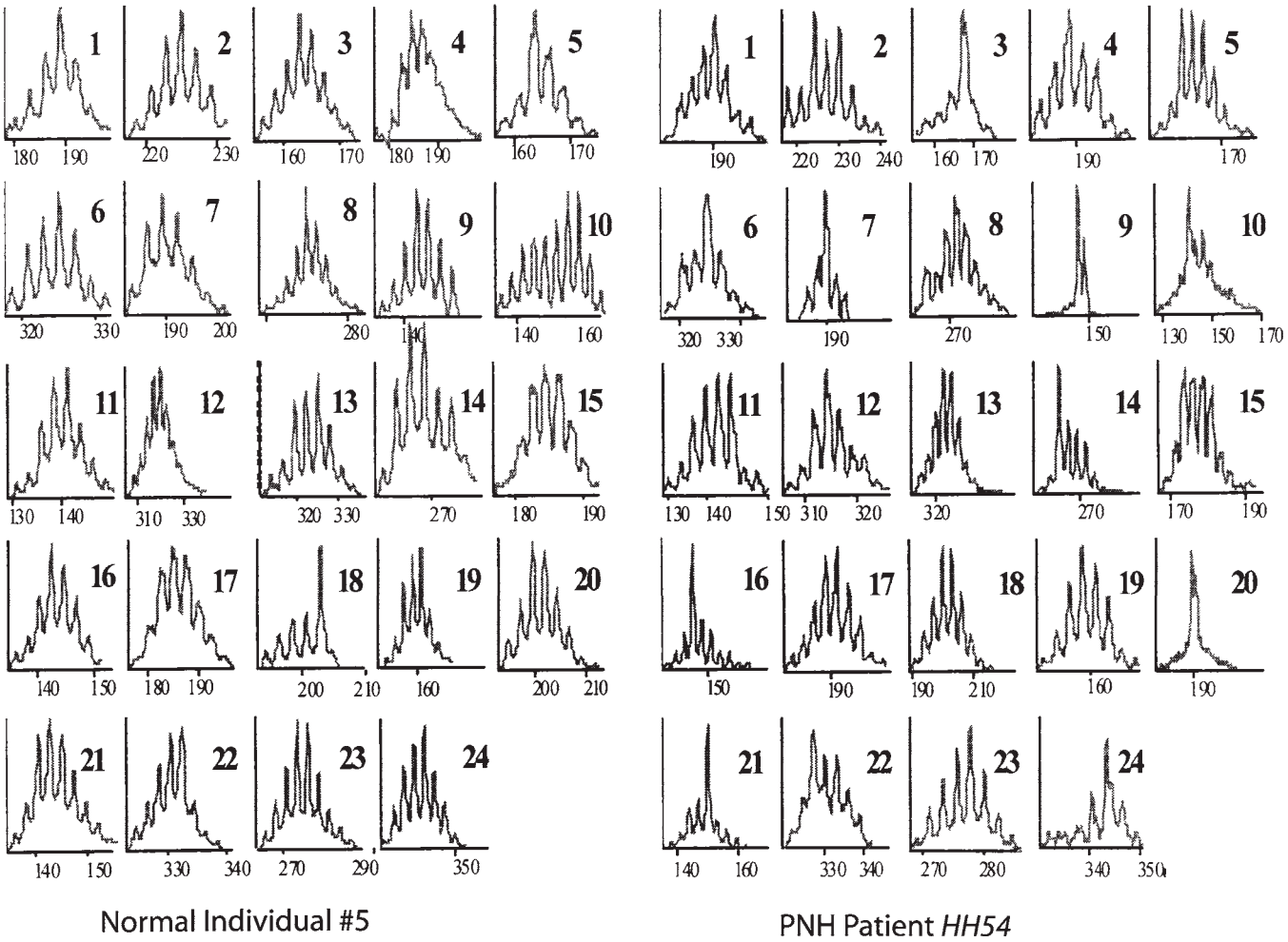

Figure 86-4 T-cell repertoire by TCR-VB CDR3 size-distribution analysis (immunoscope) in paroxysmal nocturnal hemoglobinuria (PNH) patients. The complementary determining region 3 (CDR3) mRNA pool is amplified by reverse transcriptase-PCR using a fluorescent constant-B primer and the specific variable-B (VB) primer. The complexity of the VB-CDR3 repertoire is resolved on acrylamide gel and displayed as relative fluorescence intensity (vertical axis): peaks represent mRNAs of identical length (horizontal axis). In healthy individuals, the normally diverse repertoire consists of 6–10 peaks, 3 nucleotides distanced from each other (representing in-frame functional rearrangements), with a bell-shaped (Gaussian like) distribution. In PNH, T-cell oligoclonality results in the appearance of prominent peaks out of the bell (skewing); in this paradigmatic PNH patient, VB3, VB7, VB9, VB14, VB16, VB20, VB21, and VB24 are skewed. (Reproduced with permission from Karadimitris A, Manavalan JS, Thaler HT, et al. Abnormal T-cell repertoire is consistent with immune process underlying the pathogenesis of paroxysmal nocturnal hemoglobinuria. Blood 2000;96:2613–2620. © American Society of Hematology.)

PNH with AA, the increased incidence of PNH in countries where AA is more frequent, as well as the frequent association of both diseases with human leukocyte antigens known to be related to autoimmune disorders.

2. The PNH clones, although responsible for a number of clinical consequences, are not invasive; rather, they should be considered "salvage" clones, which positively mitigate the consequence of bone marrow failure. Indeed, the gene expression profile of CD34+ PNH cells as studied by a microarray technique is similar to that of CD34+ cells from normal individuals, whereas phenotypically normal CD34+ cells sorted from PNH patients showed overexpression of genes possibly related to immune-mediated damage, such as TRAIL, interferon-γ, p53, and caspase 3, as seen in AA patients.
3. The long survival of patients with PNH demonstrates that a single stem cell may support most of hematopoiesis for several years. The same single *PIG-A* gene mutation detected at diagnosis (often on a banked specimen) was present after 6–10 yr of disease course. However, additional clones may develop, as seen in a patient relapsing after syngeneic bone marrow transplant who showed a *PIG-A* mutation different from that occurring at diagnosis.
4. The aim of treatment should be to recover normal hematopoiesis rather than to destroy or to repair the PNH clone.

CLINICAL FEATURES

The disease is rare (prevalence approx 1–2/million), but it occurs throughout the world, with increased prevalence in parts of Asia (e.g., Thailand, China), in which unexplained higher incidence of AA is also observed. PNH is characterized by chronic hemolytic anemia with erratic exacerbations, frequent leukopenia and thrombocytopenia, tendency to thrombosis, and, to a lesser extent, to infection. The clinical picture can range from mild to severe, severity being dictated by extreme pancytopenia (the PNH/AA syndrome), hyperhemolysis, or recurrent thrombosis.

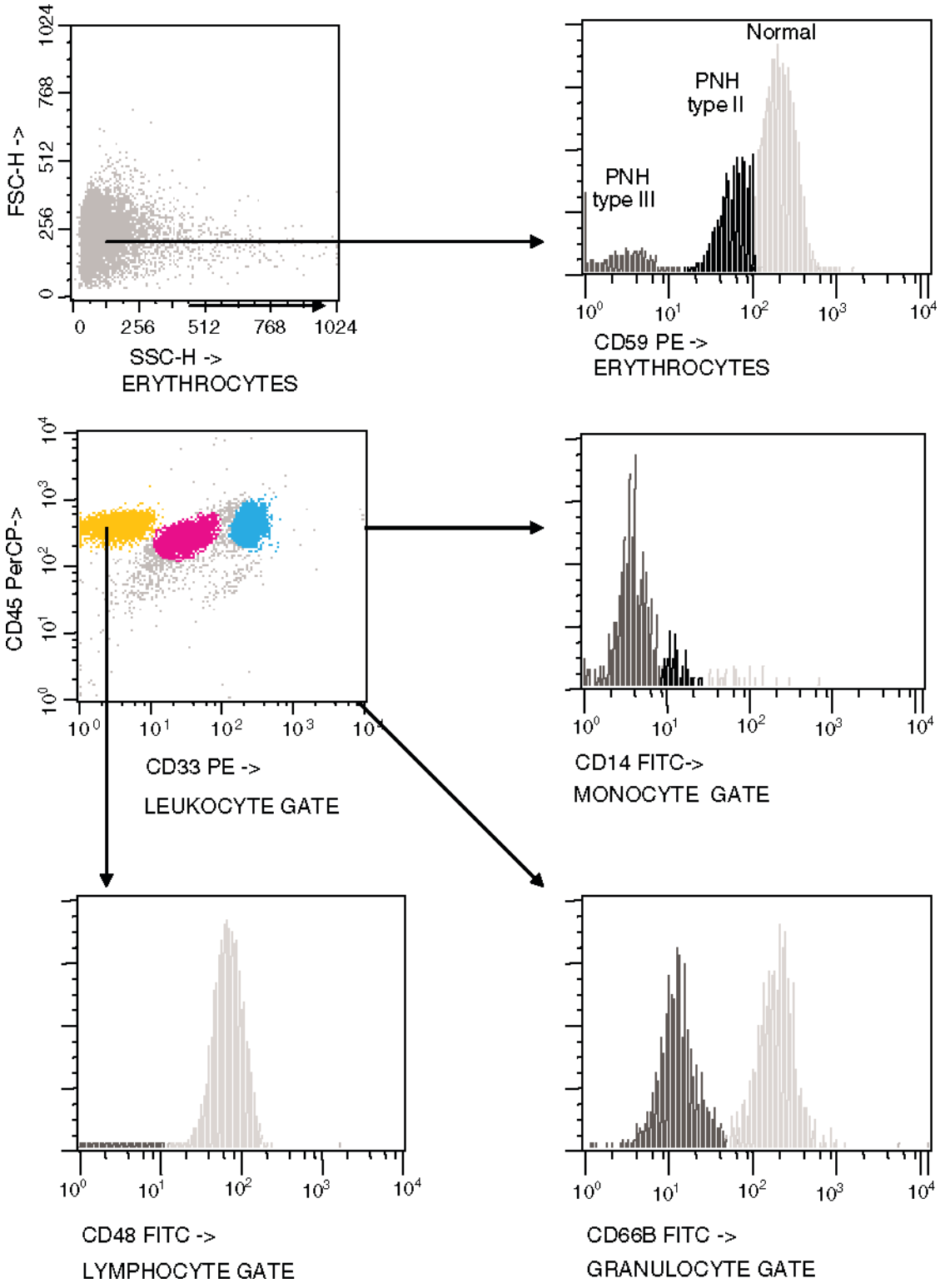

Figure 86-5 Blood cell flow cytometry profile in a representative paroxysmal nocturnal hemoglobinuria patient. (**Upper panels**): ungated red cells stained with anti-CD59 monoclonal antibody (mAb), showing three RBC populations, one normal, a second partially CD59 deficient (PNH II) and a third, completely CD59 deficient (PNH III). (**Middle left panel**): WBC are gated into three discrete populations by the combination of anti-CD45 and anti-CD33 mAbs. Granulocytes, stained with an anti-CD66b mAb, show two well-cut populations, one normal, and the other CD66b deficient; monocytes, stained with an anti-CD14 mAb, show a major population completely CD14 deficient and two smaller populations (one partially deficient and few normal cells); lymphocytes stained with an anti-CD48 mAb show a single peak of positive cells.

Although the disease is not malignant (progression to acute leukemia is rare, and the frequency may have been overestimated), with a median survival of 10 yr in large series, the course may be stormy and the quality of life poor. The major cause of death is thrombosis, often occurring in sites unusual for patients with other types of thrombophilia (suprahepatic veins leading to Budd-Chiari

syndrome, mesenteric veins, cerebral veins). The mechanisms leading to the thrombophilic state in PNH are poorly understood. They include platelet activation resulting from membrane hypersensitivity to activated complement, thrombogenic properties of microvesicles released by red cells during hyperhemolysis and by the endothelium, and fibrinolytic system impairment owing to lack of membrane-bound and excess of soluble urokinase plasminogen activator. The risk of thrombosis correlates with the PNH clone size, thus being higher in patients with the hemolytic type of disease (as opposed to the aplastic type).

DIAGNOSIS

A mild jaundice without splenic enlargement, anemia with moderate reticulocytosis, elevated serum lactate dehydrogenase, episodes of dark urine, and constant hemosiderinuria are clues for diagnosis. Until the late 1980s, the only confirmatory test was the Ham test (lysis of a portion of red cells in acidified serum); simpler but less specific tests were the sucrose and sugar–water tests (red cells' lysis in serum with reduced ionic strength). Today, flow cytometry analysis of blood cells represents the most sensitive and specific diagnostic test and has rendered obsolete the Ham test. Several GPI-anchored proteins (GPI-APs) are expressed on different blood cells, and fluorochrome-conjugated monoclonal antibodies specific to each protein are available for routine diagnostic test. Simultaneous multiparameter cytometers allow accurate detection of GPI-AP deficient populations, measuring their extent within each cell lineage. By this technique, one or two populations with abnormal expression of GPI-AP may be shown: one completely lacking GPI-AP expression (type-III PNH cells), and another characterized by their faint (dim) expression (type-II PNH cells) (Fig. 86-5). The simultaneous absence of different GPI-linked proteins on the same cells validates the specificity of the test. Flow cytometry analysis allows detection of even small PNH clones (<1% of the tested cell population). Molecular studies on DNA or mRNA to identify the specific mutation within the *PIG-A* gene are fully confirmatory tests; however, they do not add clinically informative data and are usually limited to research purposes.

TREATMENT

The treatment of PNH is essentially supportive. Prevention and early treatment of concurrent infectious or inflammatory diseases may help in reducing the frequency and severity of paroxysmal exacerbations. Folate supplement and red cell transfusions are the basis for ameliorating anemia. Iron supplement (to balance the persistent urinary loss of iron in the form of hemosiderinuria) or iron chelation (if iron overload develops because of frequent transfusions) may be needed in selected patients. Anticoagulants are used to treat or prevent thrombosis.

Based on the escape theory and on the likelihood that the mechanism inhibiting the normal hematopoiesis is immune-mediated, as in most cases of AA, immunosuppressive regimens with antilymphocyte or antithymocyte globulin associated with high-dose prednisone and cyclosporin A have been used, with variable results. Stem cell transplant approaches have been tried. Autologous transplant is precluded by a marked deficiency of normal progenitor cells in PNH bone marrow, as already suggested by a poor growth of hematopoietic progenitors in vitro; also, attempts to mobilize a sufficient number of stem cells by growth factors have failed. Allogeneic stem cell transplant from a human leukocyte antigen-matched sibling is the best option for patients younger than 45 yr, with a cure rate of approx 50%. Allogeneic transplants in older patients or from matched unrelated donors in young patients carry a high transplant-related mortality, which can be tolerated only if the quality of life of the patient is poor. If the escape theory is correct, there is little room for futuristic approaches with cell therapy (insertion of molecules on the outer surface of blood cells) or with gene therapy (insertion of a functional *PIG-A* gene in early hematopoietic progenitors) in PNH, because a repair of the damaged cell may result in cell destruction. In most PNH patients, a residual population of normal hematopoietic progenitors already exists, but it is unable to repopulate bone marrow.

A supportive treatment has been envisaged by using a monoclonal antibody targeting the fraction C5 of the complement cascade eculizumab; by blocking the formation of the terminal membrane attack complex, both chronic and paroxysmal hemolysis can be reduced, with favorable clinical consequences at least concerning hyperhemolysis (decreased lactate dehydrogenase, reduced transfusion requirement), even if the PNH population remains unchanged or even increases.

FUTURE DIRECTIONS

Although many pathophysiological aspects of PNH have been unraveled, several important items require clarification, such as:

1. The functional properties of the GPI-anchoring system in human cells.
2. The structure and the function of the protein encoded by *PIG-A*.
3. The exact role of each GPI-linked molecule on blood cell development and function.
4. The amount of transfer of GPI-linked molecules that may take place in vivo from one cell to another.
5. The hierarchy by which the various molecules are preferred in the cell for anchor attachment, when a reduced amount of anchor is produced.
6. The reason for the nocturnal exacerbation of hemolysis.

Also, a formal proof of the escape theory is needed. If confirmed, PNH could be regarded as a special type of nonneoplastic clonal disease, in which the expansion of the mutated cell results from a survival advantage rather than a growth advantage.

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SELECTED REFERENCES

Alfinito F, Del Vecchio L, Rocco S, Boccuni P, Musto P, Rotoli B. Blood cell flow cytometry in paroxysmal nocturnal hemoglobinuria: A tool for measuring the extent of the PNH clone. Leukemia 1996;10:1326–1330.

Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. Proc Natl Acad Sci USA 1999;96:5209–5214.

Bessler M, Hillmen P, Longo L, Luzzatto L, Mason PJ. Genomic organization of the X-linked gene (PIG-A) that is mutated in paroxysmal nocturnal haemoglobinuria and of a related autosomal pseudogene mapped to 12q21. Hum Mol Genet 1994;3:751–757.

Chen G, Kirby M, Zeng W, Young NS, Maciejewski JP. Superior growth of glycophosphatidylinositol-anchored protein-deficient progenitor cells in vitro is due to the higher apoptotic rate of progenitors with normal phenotype in vivo. Exp Hematol 2002;30:774–782.

Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1995;333: 1253–1258.

Hillmen P, Hall C, Marsh JC, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med 2004;350:552–559.

Hugel B, Socie G, Vu T, et al. Elevated levels of circulating procoagulant microparticles in patients with paroxysmal nocturnal hemoglobinuria and aplastic anemia. Blood 1999;93:3451–3456.

Jasinski M, Keller P, Fujiwara Y, Orkin SH, Bessler M. GATA1-Cre mediates Piga gene inactivation in the erythroid/megakaryocytic lineage and leads to circulating red cells with a partial deficiency in glycosyl phosphatidylinositol-linked proteins (paroxysmal nocturnal hemoglobinuria type II cells). Blood 2001;98:2248–2255.

Kawagoe K, Kitamura D, Okabe M, et al. Glycosylphosphatidylinositol-anchor-deficient mice: Implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. Blood 1996;87:3600–3606.

Keller P, Payne JL, Tremml G, et al. FES-Cre targets phosphatidylinositol glycan class A (PIGA) inactivation to hematopoietic stem cells in the bone marrow. J Exp Med 2001;194:581–589.

Luzzatto L. Paroxysmal murine Hemoglobinuria(?): A model for human PNH. Blood 1999;94:2941–2944.

Luzzatto L, Bessler M, Rotoli B. Somatic mutations in paroxysmal nocturnal hemoglobinuria: A blessing in disguise? Cell 1997;88:1–4.

Maciejewski JP, Follmann D, Nakamura R, et al. Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome. Blood 2001;98:3513–3519.

Meletis J, Terpos E. Recent insights into the pathophysiology of paroxysmal nocturnal hemoglobinuria. Med Sci Monit 2003;9:161–172.

Miyata T, Takeda J, Iida Y, et al. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science 1993;259:1318–1320.

Murakami Y, Kosaka H, Maeda Y, et al. Inefficient response of T lymphocytes to glycosylphosphatidylinositol anchor-negative cells: Implications for paroxysmal nocturnal hemoglobinuria. Blood 2002;100:4116–4122.

Nafa K, Mason PJ, Hillmen P, Luzzatto L, Bessler M. Mutations in the PIG-A gene causing paroxysmal nocturnal hemoglobinuria are mainly of the frameshift type. Blood 1995;86:4650–4655.

Nagakura S, Ishihara S, Dunn DE, et al. Decreased susceptibility of leukemic cells with PIG-A mutation to natural killer cells in vitro. Blood 2002;100:1031–1037.

Ninomiya H, Kawashima Y, Hasegawa Y, Nagasawa T. Complement-induced procoagulant alteration of red blood cell membranes with microvesicle formation in paroxysmal nocturnal haemoglobinuria (PNH): Implication for thrombogenesis in PNH. Br J Haematol 1999;106:224–231.

Nishimura Ji J, Hirota T, Kanakura Y, et al. Long-term support of hematopoiesis by a single stem cell clone in patients with paroxysmal nocturnal hemoglobinuria. Blood 2002;99:2748–2751.

Oni SB, Osunkoya BO, Luzzatto L. Paroxysmal nocturnal hemoglobinuria: Evidence for monoclonal origin of abnormal red cells. Blood 1970;36:145–152.

Plasilova M, Risitano AM, O'Keefe CL, Rodriguez A, Wlodarski M, Maciejewski JP. Shared and individual specificities of immunodominant cytotoxic T cell clones in paroxysmal nocturnal hemoglobinuria as determined by molecular analysis. Exp Hematol 2004;32:261–269.

Rawstron AC, Rollinson SJ, Richards S, et al. The PNH phenotype cells that emerge in most patients after CAMPATH-1H therapy are present prior to treatment. Br J Haematol 1999;107:148–153.

Risitano AM, Maciejewski JP, Green S, Plasilova M, Zeng W, Young NS. In vivo dominant immune responses in aplastic anemia patients: Molecular tracking of putatively pathogenic T cells by TCRβ-CDR3 sequencing. Lancet 2004;364:353–363.

Rosse WF, Dacie JV. Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody. J Clin Invest 1966;45:736–748.

Rosse WF, Ware RE. The molecular basis of paroxysmal nocturnal hemoglobinuria. Blood 1995;86:3277–3286.

Rosti V, Tremml G, Soares V, Pandolfi PP, Luzzatto L, Bessler M. Murine embryonic stem cells without pig-a gene activity are competent for hematopoiesis with the PNH phenotype but not for clonal expansion. J Clin Invest 1997;100:1028–1036.

Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. Baillieres Clin Haematol 1989;2:113–138.

Rotoli B, Robledo R, Scarpato N, Luzzatto L. Two populations of erythroid cell progenitors in paroxysmal nocturnal hemoglobinuria. Blood 1984;64:847–851.

Saso R, Marsh J, Cevreska L, et al. Bone marrow transplants for paroxysmal nocturnal haemoglobinuria. Br J Haematol 1999;104:392–396.

Simak J, Holada K, Risitano AM, Zivny JH, Young NS, Vostal JG. Elevated counts of circulating endothelial membrane microparticles in paroxysmal nocturnal hemoglobinuria indicate inflammatory status and ongoing stimulation of vascular endothelium. Br J Hemat 2004;125:804–813.

van der Schoot CE, Huizinga TW, van 't Veer-Korthof ET, Wijmans R, Pinkster J, von dem Borne AE. Deficiency of glycosyl-phosphatidylinositol-linked membrane glycoproteins of leukocytes in paroxysmal nocturnal hemoglobinuria, description of a new diagnostic cytofluorometric assay. Blood 1990;76:1853–1859.

Yamashina M, Ueda E, Kinoshita T, et al. Inherited complete deficiency of 20-kilodalton homologous restriction factor (CD59) as a cause of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1990;323:1184–1189.

Yomtovian R, Prince GM, Medof ME. The molecular basis for paroxysmal nocturnal hemoglobinuria. Transfusion 1993;33:852–873.

Zeng W, Chen G, Kajigaya S, et al. Gene expression profiling in CD34 cells to identify differences between aplastic anemia patients and healthy volunteers. Blood 2004;103:325–332.