Paroxysmal nocturnal hemoglobinuria and myelodysplastic sydromes: clonal expansion of PIG-A-mutant hematopoietic cells in bone marrow failure

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Clones of paroxysmal nocturnal hemoglobinuria (PNH) cells – glycosylphosophoinositol (GPI)-anchored protein-deficient hematopoietic cells – can be detected by flow cytometry in patients with myelodysplastic sydromes (MDS). While the etiology of PNH is known to be a somatic mutation in the X-linked *PIG-A* gene, which abrogates GPI synthesis, the pathophysiology of PNH clonal expansion is not well understood. In frank PNH with clinical symptoms and signs of intravascular hemolysis and venous thrombosis, PNH cells dominate in the peripheral blood. Very small PNH clones can also be detected efficiently and routinely in patients with aplastic anemia and MDS. PNH clones emerge almost always in the setting of marrow failure, presumably immune-mediated hematopoietic destruction. Possible mechanisms for clonal expansion and clinical implications for the diagnosis, prognosis, and treatment of MDS are discussed.

A short history of paroxysmal nocturnal hemoglobinuria

PNH, despite its rarity, has intrigued physicians since its description in the 19th century.[1-3] Progressive understanding of the disease has paralleled advances in biochemistry, cell biology, and molecular biology; as problems in etiology and pathophysiology have been resolved, however, new questions have arisen. In the current era, treatments beyond simple blood transfusion have proven effective, particularly stem cell transplantation and the novel monoclonal antibody eculizumab; as both are expensive and entail risk, a clear description of the pathophysiology of PNH has become more important.

Early in the history of the study of PNH, interest centered on the episodic and often dramatic appearance in the morning urine of an abundant red pigment, rather than red blood cells as occurs in the far more prevalent renal pathologic processes. Once biochemical investigations identified this pigment as hemoglobin, the focus became the origin of the intravascular hemolysis that released erythrocyte contents directly into the circulation. Unlike in the more common extravascular hemolysis of transfusion reactions and in autoimmune hemolytic anemia, an antibody could not be implicated. While the erythrocytes themselves seemed defective, this intrinsic susceptibility to lysis *in vitro* could only be elicited in the presence of a non-immunoglobulin component of serum. Ham discovered that the serum component was complement, thus creating a laboratory assay for PNH cells and also initiating further research on the specific complement reaction responsible as well as the erythrocyte defect. Multiple proteins were missing from the red blood cell surface; some [decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59)] acted to inactivate complement, explaining the intravascular hemolysis. Many of these proteins were missing from white blood cells and platelets, and Luzzatto, using glucose-6-phosphate dehydrogenase heterozygosity in two female PNH patients, established the clonal origin of the defect in a hematopoietic stem cell. The nature of an apparently acquired defect that would affect expression of large numbers of unrelated proteins was obscure until the finding that all shared a peculiar linkage structure to the cell's membrane surface, the GPI anchor. Much was known about this glycolipid moiety, as it is the dominant mode of attachment of protozoan proteins, including the steps in its synthesis and subsequent covalent bonding to the carboxyl terminus of proteins destined by sequence to undergo GPI transamidation, direction to the Golgi apparatus, and cell surface expression. Kinoshita successfully identified the defect in PNH as mutations in the *PIG-A* gene, which interrupt the first step in GPI synthesis. *PIG-A*, but not any of the other genes involved in GPI production, is X-linked and, therefore, a single, acquired genetic event suffices to create a PNH stem cell. From laborious analysis of circulating granulocytes and of marrow progenitors in normal individuals, both GPI-anchored protein deficiency and PIG-A mutations can be identified in very rare cells from healthy volunteers.

Etiology is not pathophysiology

We understand at a remarkable level of detail the unusual etiology of this rare blood disease: an acquired mutation in the *PIG-A* gene, occurring in an hematopoietic stem cell, blocks synthesis of the GPI anchor and, therefore, the cell surface expression of GPI-anchored proteins on the affected cell's progeny. Absence of CD59 in particular explains the intravascular hemolysis, due to failure to inactivate the late components of complement that under physiological conditions are continuously deposited on erythrocytes. The central role of complement in red cell lysis has been dramatically illustrated by the success of eculizumab, a monoclonal antibody to C5, in most patients; eculizumab abrogates intravascular hemolysis and improves transfusion-dependence, anemia, and quality of life (although in a few cases it also reveals a previously unappreciated extravascular hemolysis, likely due to C3 binding and targeting of red cells to the reticuloendothelial system).

Nevertheless, many questions remain unanswered concerning PNH. Some date back many decades. For example, why does intravascular hemolysis occur in many patients at night? What are the factors in general that precipitate paroxysms? What distinguishes classical episodic PNH from the perhaps more typical cases in which red cell destruction is constant and low grade. The major cause of death in PNH is a peculiar type of thrombosis, which affects large veins in the abdomen and cranium. Is this deadly proclivity secondary to the release of thrombogenic erythrocyte contents into the circulation or due to the inappropriate presence or

absence of a specific GPI-anchored protein? At the level of cell biology, do PNH clones persist for decades, and how does a single clone sustain virtually all hematopoietic activity? At the cellular level, what is the fate of all the proteins that are destined to be GPI-anchored but produced in an anchorless environment-are they degraded, digested, secreted or shed from the cell, and with what consequences? For what reason has evolution preserved the GPI-anchor for certain proteins? Indeed, we have little information on the biophysical differences and the functional consequences that result from a GPI-anchor attachment compared to the more conventional transmembrane configuration. GPI-anchored proteins attach via a "fatty foot" to the surface but do not extend internally beyond the cell's membrane; they associate in cholesterol-rich, detergent-insoluble lipid raft structures and presumably must cooperate with other proteins in order to convey an internal signal after engaging a ligand.

Paroxysmal nocturnal hemoglobinuria and bone marrow failure

Perhaps the major focus of current research in PNH is the relationship of PNH and marrow failure in patients and the associated basic problem of PNH clonal expansion. Clinically, PNH has a strong relationship with aplastic anemia. Historically, some patients were recognized to have a mixture of the two diseases, manifesting both marrow failure and intravascular hemolysis. Many patients with PNH have low blood counts that cannot be attributed to peripheral destruction of cells and many younger PNH patients develop aplastic anemia. In patients first diagnosed with acquired aplastic anemia, PNH sometimes appeared to evolve after immunosuppressive treatment.

The replacement of the cumbersome Ham test with flow cytometric assays for GPI-anchored proteins greatly simplified and quantified diagnostic testing for PNH, and also allowed a better description of the relationship between PNH and aplastic anemia. By flow cytometry investigation of granulocytes, expanded clones of PNH cells were observed in a large proportion of patients with aplastic anemia at the time of their presentation; if they recovered, the clone was apparent also in erythrocytes, having been obscured earlier by transfused blood cells. As described above, minute numbers of PNH granulocytes are present in normal individuals. At the time of diagnosis of aplastic anemia, between one third and one half of cases have elevated numbers of PNH granulocytes, but the proportion is usually much lower – just a small percentage of the total neutrophils – than in classical hemolytic PNH, in which the proportion of erythrocytes and granulocytes that lack GPI-anchored proteins is high, usually above 50%. In aplastic anemia the small clone may be stable in size for years, and in only a minority of cases does it increase to levels associated with frank hemolysis and anemia.

The problem of clonal expansion

The *PIG-A* mutation alone is necessary but insufficient to explain PNH clonal expansion. In humans, *PIG-A* mutant cells occur normally while PNH is a rare disease. In mouse knock-out models, absence of a functioning *Pig-a* gene in chimeric animals leads to small clones that do not increase over time, seemingly lacking a survival or proliferative advantage over normal cells. The strong association of PNH with aplastic anemia, in which an immune pathophysiology has been inferred from clinical success with immunosuppressive therapies and laboratory studies supporting a T-cell mechanism of marrow destruction, has pointed to a possible immune mechanism of selection. Findings in favor of this hypothesis are that PNH has been linked to specific histocompatability antigens; clones derived from different *PIG-A* mutant progenitors can co-exist or succeed one another in the same patient; and in some (but not all) series of patients, the presence of a PNH clones has been predictive of response to immunosuppressive therapies. There is some direct evidence for "clonal escape". In microarrays of fractionated human cell populations, the transcriptome of PNH phenotype CD34 cells from patients' bone marrows was similar to that of normal CD34 cells from healthy donors. In contrast, the normal phenotype cells (expressing GPI-anchored proteins) from the PNH patients showed activation of apoptosis, cell death, and immune response pathways, as in bone marrow form patients with aplastic anemia (the same patterns were seen in classic and aplastic PNH, suggesting a common pathophysiology).[4]

Nevertheless, no satisfying molecular mechanism has been well supported by results of *in vitro* experiments, and immune escape does not have good clinical correlates. In tissue culture, PNH cells have generally been equivalent to normal cells in immunological assays that test their ability to stimulate an immune response and their adequacy as targets of immune-mediated cytotoxicity. In patients, PNH clone size does not vary much during recovery of hematopoiesis or relapse of cytopenias, nor with application or discontinuation of immunosuppressive therapy. Allogeneic transplantation for PNH has provided opportunities for *in vivo* observations. PNH clonal emergence was seen in a patient who rejected a graft,[5] but both normal and PNH cells decline concurrently with donor immune system engraftment and are equally good targets of T-cell attack *in vitro*.[6]

An alternative relationship between PNH clonal expansion has been suggested by observations of T-cell oligoclonality in patients and *Pig-a* mutant mice. In a recent publication, Notaro and colleagues described oligoclonal expansion of cytotoxic T cells, belonging to the same T-cell receptor V-β subfamily; sharing of clonotypes, or the sequence of the antigen-binding region of the T-cell receptor, despite histocompatability antigen differences, was interpreted as the result of a (non-protein) antigen-driven process common to PNH patients-possibly to the GPI anchor itself.[7] In the murine system, we observed marked expansion of specific CD8 cell oligoclones in the marrow and spleen of conditional knock-out animals, reproduced even more dramatically after transplantation; these T cells were neither activated nor associated with active marrow failure and thus appeared to result directly from the induced mutation.[8] Thus the link between PNH and the immune system remains a tantalizing but unconfirmed possibility.

Paroxysmal nocturnal hemoglobinuria clonal expansion and myelodysplastic syndromes

PNH clones can also occur in MDS. This finding is not surprising in the context of a close and sometimes puzzling relationship between aplastic anemia and MDS. Cytopenias, not leukemia, are the most common manifestation of MDS, and the distinction between aplastic anemia and hypoplastic MDS in the individual patient may be problematic when based solely on subjective criteria of marrow morphology applied to a scanty tissue specimen. About 20% of MDS cases have hypocellular marrows, morphological criteria remain qualitative, and cytogenetics may be unobtainable or subject to interpretation.[9] Furthermore, aplastic anemia can evolve into MDS, with acquisition of typical morphological abnormalities and stereotypical cytogenetic aberrations, especially monosomy 7 and trisomy 8. There are also shared pathophysiological features; for example, trisomy 8 MDS features oligoclonal T-cell expansion and responsiveness to treatment with immunosuppressive drugs.

We first described small clones in about 20% of patients referred to the NIH Clinical Center with a diagnosis of MDS and presentation with marrow failure.[10] Several other groups confirmed these findings. However, while there is agreement that PNH clonal expansion occurs in some MDS patients, there is disagreement on several important issues: (i) which type of MDS is associated with PNH; (ii) is there prognostic significance and predictive value for therapy outcomes; (iii) how are PNH cells best measured in the routine laboratory and (iv) the pathophysiological interpretation of PNH clones in MDS as they relate to marrow failure and to leukemic transformation.

The report by Wang *et al*. in this issue of the journal[11] concerns the largest series of patients systematically and prospectively tested by flow cytometry for PNH. The study has many advantages: it originates in a center for the diagnosis and management of MDS, lending security to the clinical information; in the cytometry laboratory, special care was taken to gate on the neutrophil population; and the investigation of four GPI-anchored proteins provided a stringent measure of a putative PNH clone. The disadvantages of this study, appropriately acknowledged by the authors, were the utilization of the aerolysin method, which is not standard in commercial laboratories; absence of molecular data that PIG-A was mutated in the GPI-anchored protein-deficient cells; and the inability to correlate the presence of clones with clinical outcomes. Nevertheless, the data produced by Wang *et al*. can be used to address some issues related to PNH clones in MDS.

Myelodysplastic syndromes subtypes and paroxysmal nocturnal hemoglobinuria

In the series reported by Wang *et al*., 35% of the patients with refractory anemia harbored a PNH clone and 20% of patients with 5q- also showed clonal expansion; granulocytes deficient in multiple GPI-anchored proteins were not, however, detected in patients with ringed sideroblastic anemia, refractory anemia with excess blasts or with a diagnosis of frank acute myeloid leukemia, nor in any of the few patients tested who had a cross-over MDS syndrome, MDS with myeloproliferative disease or MDS with myelofibrosis. Thus, most published studies now agree that PNH is more likely to be detected in MDS patients who present with marrow failure, whose disease is less likely to transform into leukemia, and who, therefore, have a better prognosis; refractory anemia has been the most common subtype associated with clonal expansion.[10,12,13,14]

Paroxysmal nocturnal hemoglobinuria clones and the prognosis of myelodysplastic syndromes

As mentioned above, the cohort of patients studied by Wang *et al*. did not provide information concerning the prognostic significance of the presence of a PNH clone in MDS. Confounding retrospective analysis of other studies is the association of PNH and good prognosis MDS, and the insufficient number of patients in most previous series to make strong claims for PNH clone testing in the clinic. In a series of Japanese patients, within the refractory anemia category the presence of even a very small PNH clone (in about 20% of patients) conferred a better prognosis, less frequent transformation to leukemia, and a much higher response rate to cyclosporine.[14] In contrast, in a large group of NIH MDS patients who were mostly in low risk subgroups, the concurrent presence of a PNH clone was not associated with likelihood of response to immunosuppression or with a survival advantage.[15] Even in aplastic anemia, a more homogenous disease than MDS which has been historically associated with PNH, and in which, therefore, large numbers of patients have been and are routinely assessed for PNH, the prognostic value of finding a clone remains controversial.

Paroxysmal nocturnal hemoglobinuria testing in the clinical laboratory

Wang *et al*. appropriately devote discussion to the specifics of their flow cytometric analysis, and especially the importance of gating for granulocytes and of excluding monocytes, the necessity of multiple GPI-anchored proteins as targets for staining with monoclonal antibodies, and the physiological and pathological differences in the cell surface expression of specific GPI-anchored proteins, due to stage of differentiation under normal conditions and aberrant protein expression that occurs in MDS and leukemia. Focusing on inherently abnormal myeloid cells of chronic myelomonocytic leukemia would provide an explanation for the single report of an apparently high prevalence of PNH in this syndrome, formerly considered a MDS but now classified with the myeloproliferative diseases.[16]

Paroxysmal nocturnal hemoglobinuria clones and the pathophysiology of myelodysplastic syndromes

MDS is the result of clonal selection, and as in malignant transformation in general, many genetic changes have been described which result in enhanced proliferation, decreased cell death, and failure to undergo normal differentiation.[17,18] In a few instances, more specific selective mechanisms have been identified; as one

example, evolution to monosomy 7 has been linked clinically to G-CSF use and, in the laboratory, to expression of a G-CSF receptor that signals for proliferation but not differentiation.[19] The active selective factor for PNH in MDS, as for PNH in association with aplastic anemia, is presumed to be altered immunity, but there are no convincing laboratory data to specify a mechanism. Clinical observations only indirectly support an immunological basis for selection: PNH clones in MDS are similarly associated (as in aplastic anemia) with multiple mutant *PIG-A*-deficient cells,[13,20] with HLA-DR15, and with response to immunosuppression.[14] A novel observation in the study by Wang *et al*. is the frequency of PNH clones in the 5q- syndrome, a subtype of MDS in which an immunological component has been inferred from the success of treatment with an immunomodulatory drug, lenalidomide.

How do *PIG-A* mutations and PNH clones relate to the preleukemic character of MDS? The *PIG-A* mutation does not appear to produce genomic instability in hematopoietic stem cells. While MDS or leukemia occasionally arises from within the PNH clone,[21] transformation occurs far more frequently in non-PNH cells.[22] In the laboratory, the mutation rate in PIG-A-mutant cells is normal.[23] The highly abnormal MDS marrow microenvironment likely provides diverse, unusual selection pressures, allowing the emergence of multiple abnormal populations of hematopoietic stem and progenitor cells.

Treatment implications

Occasionally PNH patients have a dysplastic bone marrow, but these rare cases are managed based on their dominant clinical features—hemolysis, thrombosis, or marrow failure. In much more frequent typical MDS, PNH clones are almost always very small and, therefore, unlikely to contribute to an anemia that is the result of failed marrow production or to increase the risk of thrombosis. As discussed, the prognostic utility of PNH clones, suggested in some studies to be positive, needs to be confirmed prospectively in other series.

A new therapy is now available to treat PNH, and physicians may be uncertain as to when to employ an effective yet expensive and not risk-free novel intervention. Eculizumab, marketed in the United States as Soliris, acts to block intravascular hemolysis.[24] Indications for its employment include anemia and symptoms related to hemolysis; frequently, patients not only have improved hemoglobin levels but also experience relief of disproportionate fatigue, and symptoms such as dysphagia and sexual dysfunction blamed on the release of erythrocyte contents and consumption of nitric oxide in the circulation. A lower rate of thrombosis, accompanying blockade of hemolysis, was inferred from retrospective analysis of treated PNH patients (not a randomized controlled trial designed with this end-point).[25]

However – importantly – the finding of a tiny PNH clone in a patient with MDS is not an indication for therapy with eculizumab, and complement blockade would not be expected to improve symptoms or long-term survival in this patient population.

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Clinical relevance of *JAK2* (V617F) mutant allele burden

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The identification of a gain-of-function mutation in the Janus kinase 2 gene, named *JAK2* (V617F), opened a new era in the understanding of Philadelphia-negative myeloproliferative neoplasms,[1,2] including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). These entities share some clinical features such as a high risk of developing thrombosis,[3] evolution into secondary myelofibrosis (for PV and ET) and transformation into leukemia.[4]

The most intriguing question that arose after the discovery of the mutation is how a single mutation might give rise to at least three different diseases. This question remains unanswered, but clinical, biological and pathological data have led to three potential hypotheses. One, called the gene-dosage hypothesis, postulates a correlation between disease phenotype and the proportion of *JAK2* (V617F) mutant alleles introducing the concept of allele burden, that is, the ratio between mutant and wild type *JAK2* in hematopoietic cells. Experiments on transgenic mice expressing variable levels of *JAK2* (V617F) support this hypothesis.[5] In fact low levels of *JAK2* (V617F) load induce an ET-like phenotype dominated by thrombocytosis, whereas higher levels of mutant alleles lead to a PV-like phenotype. A critical role of gene dosage effect is also indicated by studies on erythroid colonies. Homozygous *JAK2* (V617F) erythroid colonies are present in most patients with PV, but occur rarely in those with ET.[6]

A second hypothesis advocates the existence of a pre-*JAK2* phase in which additional somatic mutations or inherited predisposing alleles establish clonal hematopoiesis before the acquisition of *JAK2* (V617F). Thus, mutations other than *JAK2* may determine disease phenotype directly or by co-operating with *JAK2* mutations. Analysis of the X-chromosome inactivation pattern of clonality in familial cases of myeloproliferative neoplasm[7] has provided support for this hypothesis.

Finally, host genetic factors may contribute to phenotypic diversity among myeloproliferative neoplasms. This was documented in patients with PV and ET tested for genetic variation within *JAK2*, *MPL*, *EPOR*, and *GCSFR* genes using single nucleotide polymorphisms.[8] In addition, strain-specific differences in phenotype have been observed in mice transplanted with *JAK2* (V617F) transfected cells: Balb/c mice demonstrated markedly higher leukocyte counts, splenomegaly, and bone marrow reticulin fibrosis compared with C57Bl/6 mice.[9]

Likely, the three hypotheses, although explanatory individually, are not mutually exclusive. This is true for the patient whose clinical history is illustrated in Figure 1. This is the case of a 23-year old female with familial myeloproliferative neoplasm, whose father and uncle had ET (Figure 1a). The young girl, after an initial diagnosis of ET, developed PV with a *JAK2* (V617F) allele burden of 24.8% and clonal hematopoiesis, demonstrated through X-chromosome inactivation patterns (Figure 1b). She had erythrocytosis, thrombocytosis and did not display mobilization of CD34-positive cells. A few years later she developed myelofibrosis with an increase of the mutant allele burden to 63.3%, and an increase of circulating CD34-positive cells (Figure 1c). This case is in favor of a critical gene-dosage effect of *JAK2* (V617F) on disease evolution, as the increase of allele burden corresponded with the myelofibrotic transformation. However, the case also supports the role of additional pre-existing mutations inherited in a genetically predisposed individual. In fact, this is a case of familial myeloproliferative neoplasm and a low allele burden exists within a milieu of clonal hematopoiesis.

The distribution of the *JAK2* (V617F) mutation among PV, ET and PMF seems heterogeneous, as almost all patients with PV and with post-PV myelofibrosis and about half of those with ET and PMF carry the mutation. There is now a growing interest in *JAK2* (V617F) allele burden and its potential influence on disease phenotype, disease complications and evolution. The starting point for studying the clinical significance of allele burden is its correct assessment by quantitative assays. In this regard, the paper by Lippert *et al.* in this issue of the journal is of major interest.[10] Lippert *et al.*