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Recent developments in the understanding and management of paroxysmal nocturnal haemoglobinuria

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Summary

Paroxysmal nocturnal haemoglobinuria (PNH) has been recognised as a discrete disease entity since 1882. Approximately a half of patients will eventually die as a result of having PNH. Many of the symptoms of PNH, including recurrent abdominal pain, dysphagia, severe lethargy and erectile dysfunction, result from intravascular haemolysis with absorption of nitric oxide by free haemoglobin from the plasma. These symptoms, as well as the occurrence of thrombosis and aplasia, significantly affect patients' quality of life; thrombosis is the leading cause of premature mortality. The syndrome of haemolytic-anaemia-associated pulmonary hypertension has been further identified in PNH patients. There is currently an air of excitement surrounding therapies for PNH as recent therapeutic developments, particularly the use of the complement inhibitor eculizumab, promise to radically alter the symptomatology and natural history of haemolytic PNH.

Keywords: paroxysmal nocturnal haemoglobinuria, management, complement, nitric oxide, eculizumab.

Paroxysmal nocturnal haemoglobinuria (PNH)

Introduction

Paroxysmal nocturnal haemoglobinuria is characterised by intravascular haemolysis and venous thrombosis and is associated with aplastic anaemia (Hillmen *et al*, 1995). The characteristic symptoms of PNH, abdominal pain, dysphagia, erectile failure and intense lethargy, can be attributed to the intense intravascular haemolysis and the release of free plasma haemoglobin from its intra-cellular compartment (Rother *et al*, 2005). Dacie (1963) first suggested that PNH is an acquired clonal disorder resulting from a somatic mutation in a haematopoietic stem cell. The demonstration, in two glucose-6-phosphate dehydrogenase (G6PD) heterozygote women with PNH, that only one G6PD variant enzyme was present in the PNH red cells whereas both variants were present in the residual normal red cells, provided conclusive evidence of the clonal nature of PNH (Oni *et al*, 1970). It was clear by the 1980s that PNH cells were deficient in a large number of cell surface proteins, but it was unclear how this related to either the monoclonal nature of PNH or the haemolysis. The development of immortalised cell lines with the PNH abnormality (both B- and T-cells lines) facilitated the rapid elucidation of the defect (Schubert *et al*, 1990; Hillmen *et al*, 1992; Nakakuma *et al*, 1994). It became clear that a variety of proteins normally attached to the cell membrane by a glycolipid structure, were found to be abnormal, and that this was due to a disruption in the glycosylphosphatidylinositol (GPI) biosynthetic pathway in PNH cells. A single mutation disrupting GPI biosynthesis results in a deficiency of all GPI-anchored proteins on the cell membrane and in the PNH phenotype (Armstrong *et al*, 1992; Hillmen *et al*, 1993; Takahashi *et al*, 1993).

Paroxysmal nocturnal haemoglobinuria arises through a somatic mutation of the phosphatidylinositol glycan complementation class A gene (*PIGA*) in a haematopoietic stem cell followed by a tremendous expansion of this abnormal clone (Takeda *et al*, 1993). *PIGA* is located on the X chromosome while all other genes necessary for GPI biosynthesis are located on autosomes (Kinoshita *et al*, 1997). Thus, a single mutation of *PIGA* would lead to a GPI-deficient phenotype (including females due to X-inactivation) whereas, for the other genes involved in GPI biosynthesis, mutations of both alleles would be necessary to produce the GPI-deficient phenotype. This explains why mutations in *PIGA*, rather than in the other GPI biosynthetic genes, have been found in almost all PNH patients examined (Tomita, 1999; Rosti, 2000). The exception is the recent report of a congenital syndrome affecting three individuals from two separate kindreds. Patients affected with this syndrome showed a partial GPI deficiency from their cells, developed a Budd–Chiari syndrome in early childhood, and demonstrated elevated levels of lactate dehydrogenase (LDH), which was not manifest as clinically apparent haemoglobinuria. This congenital GPI deficiency is inherited in an autosomal recessive manner and results from a mutation of the PIG-M gene (*PIGM*) (Almeida *et al*, 2006).

The functions of the GPI-linked proteins are extremely varied. At least two are important in the control of comple-

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ment. Decay accelerating factor (DAF or CD55) controls the early part of the complement cascade by regulating the activity of the C3 and C5 convertases. CD59 inhibits terminal complement by preventing the incorporation of C9 onto C5b-8 and therefore preventing the formation of the membrane attack complex (MAC). As a result of complement-mediated attack, the survival of PNH erythrocytes *in vivo* is shortened to about 10% that of normal red cells (Wiedmer *et al*, 1993). Patients with red cells that have predominantly partial deficiency of CD59 (those with <10% PNH type III red cells) do not suffer from significant intravascular haemolysis indicating that low levels of this protein are sufficient to adequately block MAC assembly.

Glycosylphosphatidylinositol protein deficiency on PNH red cells can be complete (PNH type III cells) or partial (PNH type II cells). Cells with normal levels of GPI proteins on their surface are referred to as type I cells (Fujioka & Yamada, 1994). This classification of PNH red cells derives from different complement lysis sensitivities with type III and type II red cells being 15–25 times and 3–5 times more sensitive to complement than the type I normal red cells, respectively (Rosse, 1990). This variability in the severity of the deficiency, as well as in the proportion of the cell population affected, defines the extent of the clinical manifestations of the disease (Tomita, 1999; Rosti, 2000). The analysis of GPI-anchored proteins on the surface of the haematopoietic cells, particularly the red cells, in PNH reveals that approximately 40% of patients have a combination of types I, II and III PNH cells (see Fig 1).

Classification of PNH

The development of flow cytometry has allowed the detection of small PNH clones which would otherwise not be identified. This has allowed their identification in a proportion of patients with aplastic anaemia and other marrow failure disorders and has led to the classification of PNH into two broad groups: (i) haemolytic PNH, characterised by overt episodes of intravascular haemolysis and typically with large PNH clones and (ii) hypoplastic PNH with a clinical picture dominated by cytopenias, with no overt haemolysis and normally small PNH clones or predominantly Type II cells (Richards *et al*, 2000). An updated classification system has recently been proposed, which divides patients with PNH clones into the following subgroups (Parker *et al*, 2005):

1 Classic PNH.
2 PNH in the setting of another specified bone marrow disorder (e.g. PNH/aplastic anaemia or PNH/refractory anaemia-myelodysplastic syndrome).
3 PNH-subclinical (PNH-sc) in the setting of another specified bone marrow disorder (e.g. PNH-sc/aplastic anaemia).

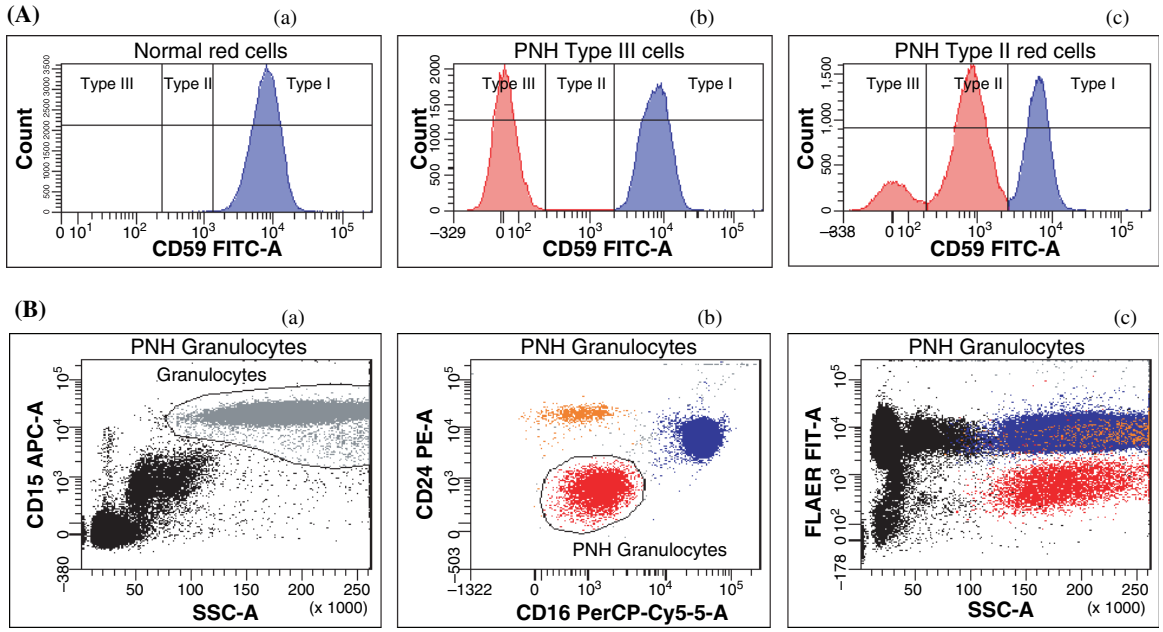

Fig 1. Flow cytometry of red cells and granulocytes: detection of paroxysmal nocturnal haemoglobinuria (PNH) clones (A) red cells. Analysis of CD59 expression by flow cytometry on (a) normal, and (b and c) PNH red cells. Histogram (a) shows normal CD59 expression (type I cells). Plot (b) shows a major population of completely CD59-deficient red cells (46%, type III cells) and a population of normal red cells. Plot (c) shows a mixture of all three types of red cells; type III, 10%; type II (partial CD59 expression), 54% and normal type I cells, 36%. (B) Granulocytes. (a) Multicolour flow cytometry for the detection of granulocyte PNH clones. Granulocytes are identified on the basis of CD15 expression and variable SSC. (b) Analysis of CD16 and CD24 [both glycosylphosphatidylinositol (GPI)-linked antigens] for this population of cells shows a small population of PNH granulocytes that is deficient for both antigens and comprises 10% of total granulocytes. (c) shows the same case also stained with a FLAER (fluorescent aerolysin), a novel reagent that binds specifically to the GPI anchor. This is also an effective reagent for the detection of PNH cells by flow cytometry and can be easily used in combination with monoclonal antibodies.

The obvious concern regarding the above classification is the overlap between haemolytic 'classic PNH', which probably always has an underlying marrow failure, even if this is not apparent clinically, and 'PNH in the setting of another specified bone marrow disorder', which may have clinically significant haemolysis.

Pathogenesis of PNH

It does not appear that GPI-deficient clones have an inherent growth advantage over their normal counterparts. So, how can PNH clones populate the haematopoietic compartment in patients with PNH? There is convincing evidence to indicate that all patients with PNH have an underlying bone marrow failure, usually aplastic anaemia, either preceding or co-existent with the diagnosis of PNH (Rotoli *et al*, 1982; Rotoli & Luzzatto, 1989; Maciejewski *et al*, 1997). This led to the conclusion that patients with PNH are permissive for the expansion of GPI-deficient haematopoietic clones. These clones do not have any 'malignant' tendency in that they appear to be regulated in a normal manner with no tendency to metastasise beyond the normal haematopoietic compartment. In fact, GPI-deficient cells with *PIGA* mutations occur very frequently at low levels in normal individuals (Araten *et al*, 1999), but do not expand in competition with the normal haematopoietic cells. They can, however, expand if there is a selective advantage for them to do so (Rawstron *et al*, 1999) as described in aplastic anaemia patients that develop PNH (Tichelli *et al*, 1988; Schrezenmeier *et al*, 1995). It appears that normal haematopoiesis is suppressed by the immune system, presumably either directly or indirectly through one or more GPI-linked proteins, and that this attack spares the GPI-deficient PNH clone. Thus, in an environment where there is intense pressure for haematopoiesis, the PNH clone is driven to produce mature haematopoietic cells and expands to fill the void left by the aplastic process. This two-hit hypothesis is further supported by some mice models of PNH, which have been generated by using targeted disruption of the PIG-A gene in mouse embryonic stem cells. These animals have a discrete proportion of blood cells devoid of GPI-linked proteins, but there are no signs of a substantial expansion of the PNH clone (Murakami *et al*, 1999; Jasinski *et al*, 2001; Rosti, 2002). This is because whilst these mouse models have a *PIG-A* deficient clone, they do not have an underlying marrow failure to favour its expansion. The exact mechanism for the relative growth advantage of PNH cells remains unclear but is the subject of considerable scientific activity. A theory previously proposed by Inoue *et al* (2003) suggested that clonal expansion might be a consequence of clonal evolution in which a second somatic mutation in the *PIGA*-mutant stem cell confers a proliferative advantage. A recent paper by this group supports this model by demonstrating ectopic expression of *HMGA2* in the *PIGA*-mutant cells of two PNH patients (Inoue *et al*, 2006).

How does the development of PNH relate to the pathogenesis of aplastic anaemia? Aplastic anaemia may be caused by a toxic insult, for example viral infections, drugs and irradiation. It is likely that bone marrow injury initiates an immune-mediated attack against a target protein on the normal stem cell, which is temporarily altered by the causative event, making the protein appear abnormal and therefore vulnerable to the stimulation of an autoimmune attack. Then, when the original insult has disappeared, this autoimmune attack can still recognise the original nascent protein. In order for the PNH clone to evade this attack, either the target protein or an accessory molecule required for the immune destruction of the stem cell by the immune cell, presumably a cytotoxic T-cell, must be GPI-linked. Therefore, the automimmune process is unable to kill the PNH stem cell efficiently. Under those circumstances, *PIGA* mutant stem cells would escape injury and consequently dominate haematopoiesis because they lack the target protein. The expansion of GPI-negative lymphocytes has been demonstrated after CAMPATH-1H therapy (Hertenstein *et al*, 1995; Taylor *et al*, 1997; Rawstron *et al*, 1999; Fracchiolla *et al*, 2001; Garland *et al*, 2005; Ruiz *et al*, 2006).

An alternative potential mechanism is that the PNH stem cells have an altered physiology compared with their normal counterparts. It is known that PNH stem cells have a different localisation compared with normal stem cells. In PNH patients with clone sizes >95% PNH neutrophils, the stem cells in the marrow are >95% PNH cells. In the peripheral blood the 'mature' stem cells (CD34+38+) will also be >95% PNH-derived whereas the earlier stem cells (CD34+38−) will be predominantly normal. This suggests that the most immature stem cells, presumably the ones destroyed by the aplastic process, have an abnormal localisation, which may be the reason or a result of their evasion from immune attack (Johnson *et al*, 1998; Tomita, 1999; Rosti, 2000). It is not clear why normal pluripotent cells should circulate selectively in the peripheral blood of these patients when they are only a small minority of the marrow precursors. Normal stem cells may be selectively released, perhaps because of a GPI-linkage deficiency involving adherence or homing, or they may have a survival advantage over PNH cells when in the peripheral blood that is not present in the marrow microenvironment. This phenomenon is marked in the majority of patients and it is intriguing to consider that it may have some relevance to the pathogenesis of PNH (Johnson *et al*, 1998; Han *et al*, 2004).

Clinical features

Paroxysmal nocturnal haemoglobinuria is an uncommon disease and is characterised by bone marrow failure, thrombosis and intravascular haemolysis. The brisk intravascular haemolysis commonly leads to haemoglobinuria, dysphagia, recurrent abdominal pain, severe lethargy and erectile failure. PNH is a chronic condition, frequently affecting young individuals, that may persist for many years and which often presents clinicians with difficult management problems. Based

on the two historical studies, the median survival in PNH was observed to be between 10 and 15 years from the time of diagnosis (Hillmen *et al*, 1995; Socie *et al*, 1996). It appears that with modern supportive measures, such as platelet transfusion, immune suppressive therapy for patients with bone marrow failure, and aggressive anticoagulation in select patients, the prognosis for patients with PNH has probably improved.

The symptoms associated with ongoing haemolysis and/or insufficient haematopoiesis have a major impact on the patient's well-being. Patients usually have acute exacerbations of haemolysis on the background of persistent lower levels of haemolysis. The acute exacerbations can occur either regularly or unpredictably, and have a further adverse impact on quality of life. Anaemia and the need for transfusions to sustain haemoglobin levels occur frequently. Haemolysis in patients with PNH can be monitored by levels of the enzyme LDH, which are frequently elevated, exceeding 20 times the upper limit of normal during severe paroxysms (Tabbara, 1992; Paquette *et al*, 1997; Rosti, 2000; Hillmen *et al*, 2004).

The most feared complication of PNH is venous thrombosis, which occurs in *c.* 50% of patients with haemolytic disease and is the cause of death in at least one-third (Hillmen *et al*, 1995; Socie *et al*, 1996). There is a predilection for the intra-abdominal and cerebral veins. The risk of thrombosis is greater in patients from Europe and the USA than in patients from the Far East (Parker *et al*, 2005). There also appears to be an increased risk of thrombosis in African-American or Latin-American patients (Araten *et al*, 2005). A possible explanation is that patients from different ethnic groups may have different additional inherited prothrombotic traits; however, no correlation between the inherited thrombophilia and thrombosis in PNH has been demonstrated (Nafa *et al*, 1996).

The cause of the thrombotic tendency in PNH is not entirely clear and may be multifactorial. It has been suggested that free plasma haemoglobin may contribute to platelet activation and thrombosis. The infusion of cross-linked haemoglobin increases platelet aggregation and adhesion *in vivo* on prothrombotic surfaces, such as an injured vessel wall (Olsen *et al*, 1996). Further, administration of free haemoglobin in healthy volunteers is associated with thrombophlebitis, demonstrating that haem can cause vascular inflammation followed by vascular obstruction *in vivo* (Simionatto *et al*, 1988).

The effect of free haemoglobin on platelet function is probably through the scavenging of nitric oxide (NO). NO has been shown to inhibit platelet aggregation, induce disaggregation of aggregated platelets and inhibit platelet adhesion through increasing cGMP levels (Radomski *et al*, 1987b; Radomski *et al*, 1987a). In fact, NO donor drugs (S-nitrothiols) that increase systemic levels of NO have been shown to inhibit platelet aggregation (Megson *et al*, 2000). Conversely, NO scavenging by haemoglobin or the reduction of NO generation by the inhibition of arginine metabolism results in an increase in platelet aggregation (Broekman *et al*, 1991; Olsen *et al*, 1996; Schafer *et al*, 2004). NO also interacts with components of the coagulation cascade to downregulate clot formation (Catani *et al*, 1998; Hugel *et al*, 1999; Kayanoki *et al*, 1999; Shao *et al*, 2001). There is also evidence that implicates the GPI-deficient platelets, which are more easily activated by complement than normal platelets. The absence of CD59 may render platelets susceptible to attack by complement, resulting in morphological changes and the release of vesiculated MAC. These vesicles or microparticles are procoagulant *in vitro* and are present at significantly elevated levels in the blood of patients with PNH. It is therefore possible that the absence of CD59 from platelets may contribute to thrombin generation and increased thrombotic risk. Alternative mechanisms have been suggested, such as deficiency of urokinase-like plasminogen activator receptor from PNH neutrophils or directly because of intravascular haemolysis with exposure of pro-thrombotic erythrocyte membrane vesicles (Wiedmer *et al*, 1993; Hugel *et al*, 1999; Rosse, 2001). One study has recently demonstrated that antiphospholipid antibody-induced complement activation and downstream signalling via C5a receptors in neutrophils leads to the induction of tissue factor, a key initiating component of the blood coagulation cascade and an enhanced procoagulant activity (Ritis *et al*, 2006).

While studies have reported a strong correlation between a larger PNH type III neutrophil clone and the occurrence of thrombosis (Hall *et al*, 2003; Moyo *et al*, 2004), thrombosis appears to also be elevated in patients with smaller clones as low as 10% when compared with the normal population (Hall *et al*, 2003). Hall *et al* (2003) reported that approximately 44% of patients with large PNH clones, defined by greater than half of the patient's neutrophils originating from the PNH clone, developed venous thrombosis in the first 10 years after diagnosis and that the rate in patients with smaller clones, as small as 10%, was approximately 5·8%, which was also markedly greater than the rate observed in a healthy population of approximately five thromboses per 10 000 patient years (Fowkes *et al*, 2003).

Progressive pancytopenia occurs in a proportion of patients with PNH. Up to 10% of patients will die from the results of aplastic anaemia associated with PNH. The management of pancytopenia in PNH patients is the same as that for patients with aplastic anaemia and this may significantly reduce the mortality from this complication.

The risk of leukaemia in PNH has been somewhat overstated in the literature because of preferential reporting in the form of case reports. The incidence of acute myeloid leukaemia (AML) in PNH appears to be similar to the risk of AML in aplastic anaemia, in the region of 5%. It appears likely that aplastic anaemia predisposes to clonal haematopoietic disorders such as AML, PNH and myelodysplastic syndrome but that the development of a PNH clone does not increase the risk of AML/myelodysplastic syndrome. Thus, the GPI-deficient phenotype is not preleukaemic.

A significant proportion of patients survive for prolonged periods (approximately 25% surviving over 25 years with blood transfusion support only) and about 15% will experience a spontaneous recovery from their PNH with no sequelae attributable to their disease (Hillmen *et al*, 1995). In almost all cases of PNH, there remains some level of normal haematopoiesis. There is evidence that the cytopenias associated with aplastic anaemia improve with prolonged follow-up, presumably because of a reduction in the underlying aplastic process. A possible hypothesis to explain the spontaneous recovery occasionally seen in PNH is that the aplastic process, which is positively selecting for PNH clones, reduces in intensity with time. At some point, the selection in favour of the PNH clone will lessen and haematopoiesis will swing towards the residual normal cells. Thus, the proportion of PNH cells will decrease and eventually disappear as the bone marrow function returns to normal.

Diagnosis of PNH

Flow cytometry continues to play a pivotal role in both the diagnosis and management of PNH, and more recently has played a critical role in monitoring responses to the novel therapeutic agent, eculizumab. Precise and accurate measurement of the GPI-deficient blood cells, particularly within the granulocyte and erythrocyte lineages, remains central to the rapid diagnosis of PNH. Furthermore, sequential studies of peripheral blood red cell and granulocyte PNH clones sizes can have a significant impact on management of individual patients (Richards *et al*, 2004). Sequential monitoring of red cell PNH clone sizes has played a key role in demonstrating the efficacy of the novel therapeutic agent, eculizumab, in the control of anaemia, transfusion requirement and haemolysis in patients with haemolytic PNH. In a pilot study of 11 patients, PNH red cell clones prior to treatment were disproportionately low when compared with their respective granulocyte clones. This difference was due to a combination of continuous haemolysis and a variable proportion of transfused normal red cells. In this initial series of patients, the mean red cell PNH clone increased from 36·7% pretreatment to 59·2% at 3 months. Seven patients achieved a sustained stable red cell PNH clone percentage equal to the size of the granulocyte PNH clone percentage and a further three reached at least 75% of the granulocyte clone size.

The relationship of free haemoglobin with NO depletion

Haemoglobin released from the PNH red cell during intravascular haemolysis can completely deplete haptoglobin, which is in place to remove free haemoglobin, resulting in its overflow into the urine and, in some cases, haemoglobinuria (Tabbara, 1992). Once the capacity of this haemoglobin scavenging protein is exceeded, consumption of endogenous NO ensues (Pohl & Lamontagne, 1991; Reiter *et al*, 2002). In addition to haemoglobin decompartmentalisation and NO scavenging, haemolysis also releases erythrocyte arginase, an enzyme that converts L-arginine, the substrate for NO synthesis, to ornithine, thereby further reducing the systemic availability of NO (Azizi *et al*, 1970; Morris *et al*, 2003; Schnog *et al*, 2004; Morris *et al*, 2005). Preliminary results indicate a profound degree of NO consumption in patients with PNH (Hill *et al*, 2006a).

NO and the symptoms of PNH

Haemolysis has been linked to smooth muscle dystonia including abdominal pain, dysphagia and erectile dysfunction (Rother *et al*, 2005). These symptoms occur more commonly in patients with large PNH clone sizes and, therefore, higher haemolytic rates (Rosse, 2000; Moyo *et al*, 2004). It has been proposed that these symptoms are associated with the excessive release of haemoglobin from the red blood cells (Rother *et al*, 2005). It has been well established that local NO deficiency is one of the major factors responsible for erectile dysfunction (Corbin *et al*, 2002). Benefit from PDE5 inhibitors in PNH patients is reported by patients to be substantially reduced when macroscopic haemoglobinuria is present. This may be explained by the greater NO consumption in PNH patients, secondary to intravascular haemolysis that is in excess of that seen in most other chronic haemolytic anaemias.

The relationship of NO depletion and free haemoglobin with pulmonary hypertension

The administration of cell-free haemoglobin solutions to normal volunteers and patients is commonly associated with a dose-dependent increase in blood pressure, both systolic and diastolic (Savitsky *et al*, 1978; Przybelski *et al*, 1996; Viele *et al*, 1997; Saxena *et al*, 1999; Carmichael *et al*, 2000; LaMuraglia *et al*, 2000; Lamy *et al*, 2000). This increase in blood pressure is reversed by the administration of the NO donor, sodium nitroprusside, confirming the importance of NO scavenging in vasoregulation (Erhart *et al*, 2000). Pulmonary arterial hypertension is an increasingly recognised complication of chronic hereditary and acquired haemolytic anaemias, including sickle cell disease (Norris *et al*, 1992; Sutton *et al*, 1994; Adedeji *et al*, 2001; Castro *et al*, 2003; Morris *et al*, 2003; Gladwin *et al*, 2004; Vichinsky, 2004). While respiratory or cardiac symptoms have often been attributed to the anaemia alone in PNH, we investigated whether the chronic haemolysis evident in PNH may also cause a rise in pulmonary pressures. Doppler echocardiographic assessments of pulmonary-artery systolic pressure were measured in patients with PNH, other haemolytic diseases and normal volunteers. Pulmonary hypertension was prospectively defined as a tricuspid regurgitant jet velocity of 2·5 m/s or greater. We found that *c.* 50% of patients with haemolytic PNH had elevated pulmonary artery systolic pressures, suggesting a much higher prevalence compared with other haemolytic anaemias (Hill *et al*, 2006b). These findings also suggest that dyspnoea, a very common disease-

related symptom in PNH, may be exacerbated by underlying pulmonary vascular pathophysiology as well as anaemia.

Conventional therapeutic strategies in PNH

The principle conventional therapy for PNH is supportive care with transfusions as required and the treatment of complications, such as thrombosis, when they occur. It is conventional to give folic acid supplementation to all patients with evidence of haemolysis, as they will have increased red cell production. Although many patients are transfusion-dependent, the chronic haemosiderinuria and haemoglobinuria may lead to severe iron deficiency and patients often also require supplementation with iron therapy.

The only curative strategy is allogeneic stem cell transplantation, but this carries a considerable risk of mortality (Saso *et al*, 1999). In view of the fact that a proportion of patients (*c.* 15%) will eventually experience a spontaneous remission of PNH (Hillmen *et al*, 1995) and with the advent of potentially effective novel therapies, stem cell transplantation should only be considered in selected cases, such as patients with a syngeneic donor or with associated bone marrow failure. In these patients, the indications for transplantation are similar to those for aplastic anaemia. Patients with cytopenias because of associated aplastic anaemia will often respond to immunosuppressive therapy with antilymphocyte globulin and/or ciclosporin.

Novel therapeutic strategies in PNH

Prevention and treatment of thrombosis. The first thrombosis in a patient with PNH heralds a significant deterioration in the patient's health and a marked worsening of the prognosis. There are no controlled clinical trials of antithrombotic regimens in PNH patients. Reports of tissue plasminogen activator for intra-abdominal venous thrombosis in PNH show that the thrombus may be cleared effectively (McMullin *et al*, 1994). The standard of care after established venous thrombosis in PNH is life-long full anticoagulation. In view of the high risk of thrombosis and that patients have decreased quality of life following the first thrombosis, selected patients could be considered for warfarin as primary prophylaxis prior to the thrombosis. Hall *et al* (2003) reported a retrospective analysis comparing 39 patients at high risk of venous thrombosis treated with warfarin as primary prophylaxis to 56 patients with similar-sized clones who were not treated with warfarin because of either patient and/or physician choice. The incidence of thrombosis at 10 years in the group of patients not on warfarin was 36·5% compared with no thromboses in the patients in the warfarin group. However, two patients in the warfarin group had a major haemorrhage with one dying as a direct result of an intracranial bleed. Despite that this study was a retrospective analysis and patients were not randomised, the results suggest that primary prophylaxis may be beneficial. There are no studies of antiplatelet drugs, such as aspirin or clodiprogrel, in PNH.

Gene therapy. As PNH is an acquired single gene disorder, it appears an attractive entity to consider for gene therapy initiatives. To consider such novel and potentially hazardous therapeutic options, we must be able to answer two vital questions: (i) which patients are destined to do poorly – we do not want to harm a patient who may spontaneously remit; and (ii) what is the mechanism for the growth advantage of PNH clones – a PNH cell which is 'corrected' to normal by the introduction of a normal *PIGA* gene will then express all the missing GPI-linked proteins and would then be expected to be selected against by the aplastic process. It must be remembered that PNH can be considered as a 'natural form' of gene therapy in that the PNH cells escape from the aplastic process by virtue of their mutated *PIGA* and therefore correcting the genetic abnormality may reverse the benefit the patient gains from having PNH haematopoiesis, resulting in no remaining haematopoiesis.

Replacement of complement regulatory proteins on PNH cells. An alternative therapeutic strategy in PNH could be to replace CD59, the deficient complement regulatory protein. If sufficient levels of CD59 could be delivered to the cell surface, complement-mediated haemolysis may be reduced without compromising the immunoprotective functions of the complement system (Sims *et al*, 1989; Wiedmer *et al*, 1993). It is recognised that even low levels of CD59 on erythrocyte membranes are sufficient to protect the cells from lysis *in vitro*. Several investigators have attempted CD59 replacement in the past. Rother *et al* (1994) used a recombinant transmembrane form of CD59 (CD59-TM) and demonstrated similar levels of protection against human complement-mediated membrane damage with equal levels of either CD59-TM or native CD59 (CD59-GPI).

We have recently reported the use of an alternative artificial glycolipid anchor (Prodaptin) to anchor CD59 (Prodaptin-CD59) into the cell membrane (Hill *et al*, 2006c). The Prodaptin modification enables exogenously administered proteins to bind to the cell membrane in a manner analogous to GPI anchors. A related modification of Prodaptin has been used previously to localise other complement inhibitors to disease sites in animal models and has been used in clinical studies (Linton *et al*, 2000; Rosti, 2000; Smith & Smith, 2001; Fraser *et al*, 2003; Pratt *et al*, 2003). Prodaptin-CD59 is expressed in *Escherichia coli* and modified using site-specific attachment of a membrane-interactive acylated, charged peptide instead of the native GPI anchor. The attachment of Prodaptin-CD59 to the red cell membrane is very rapid and the interaction appears to be effective for at least 3 d *in vitro*. Prodaptin-CD59 has been shown to restore resistance of PNH cells to complement *in vitro* and to transfer to adjacent cells spontaneously. In addition, i.v. injection of Prodaptin-CD59 effectively coated the erythrocytes of mice and protects them from lysis by human complement for at least 24 h. This study provided a proof of concept and represents a potential novel therapeutic approach for patients with PNH.

Inhibition of terminal complement with eculizumab. Complement activation is a complex cascade leading to the production of anaphylatoxins, chemotoxins and the MAC. There are three pathways which activate the cascade (Fig 2 – the classical (initiated by antigen/antibody complex), alternative (initiated by microbial membranes or immune complexes) and lectin pathways. These pathways all converge to cleave C5 into C5a, a potent anaphylatoxin, and C5b. C5a possesses various proinflammatory properties and enhances the procoagulant activity of neutrophils (Ritis *et al*, 2006). C5b is the initial molecule of terminal complement and binds C6 then C7 and then C8. C5b-8 forms the scaffold for C9 molecules, which bind to each other to form the MAC (also called the terminal complement complex). The MAC forms pores in the cell membrane and is responsible for the characteristic intravascular haemolysis in PNH.

Individuals with inherited deficiency of any of the complement molecules prior to C5 are vulnerable to both pyogenic organisms and to autoimmune disorders. In contrast, deficiency of any of the molecules after C5, remarkably, has little abnormal phenotype. The only apparent adverse effect is an increased risk of infection by encapsulated organisms, particularly *Neisseria meningitidis*, although terminal complement-deficient individuals appear to have a lower mortality from such infection when compared with complement-replete individuals (Petersen *et al*, 1979; Ross & Densen, 1984; Figueroa & Densen, 1991).

Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanised monoclonal antibody that specifically targets the complement protein C5 and prevents its cleavage (Thomas *et al*, 1996). Complement inhibition at this stage blocks the generation of C5a and the formation of C5b-9 while it preserves early complement components that are critical for the clearance of microorganisms and immune complexes (Matis & Rollins, 1995).

Results from a 12-week open-label study of eculizumab in patients with PNH demonstrated a dramatic reduction in haemolysis, a concomitant increase in the proportion of PNH type III RBCs, and an improvement in anaemia (Hillmen *et al*, 2004). The drug was given by i.v. infusion over 30 min and was well tolerated. In addition, this initial study showed a marked decrease in the rates of paroxysms and blood transfusions and an improvement in quality of life compared with the same patients prior to commencing therapy. A subsequent 1-year follow-up study demonstrated long-term efficacy and safety of eculizumab in these patients (Hill *et al*, 2005a).

Following the pilot study, a Phase III randomised, placebo-controlled trial (TRIUMPH; Transfusion Reduction Efficacy and Safety Clinical Investigation Using Eculizumab in Paroxysmal Nocturnal Hemoglobinuria) was conducted and the results have recently been reported (Hillmen *et al*, 2006a). The study investigated whether eculizumab improved anaemia, as measured by stabilised haemoglobin levels and reduced transfusion requirements in transfusion-dependent patients with PNH during 6 months of treatment. The effect of eculizumab on chronic intravascular haemolysis was demonstrated by an immediate and sustained decrease in LDH levels. At the end of the treatment period, 49% of patients in the eculizumab group (21 of 43) had levels of haemoglobin that remained above the prespecified set point (the level at which each individual patient was transfused before starting trial medication; median 77 g/l for both groups) in the absence of transfusions, whereas stabilisation of haemoglobin levels did not occur in any patient in the placebo group ($P < 0{\cdot}001$). By week 26, the median number of units of packed red cells transfused per patient was 0 in the eculizumab group and 10 in the placebo group ($P < 0{\cdot}001$). In the 6-month period before the study, the median number of units of packed red cells transfused per patient was 9·0 in the eculizumab cohort and 8·5

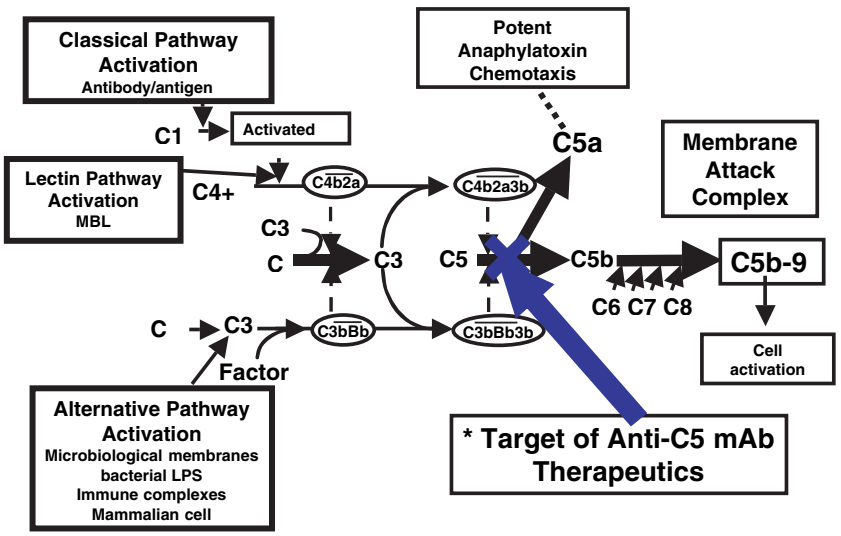

Fig 2. The complement cascade. The three pathways activating this cascade are demonstrated. All three pathways converge at C5, following which the membrane attack complex is formed that results in lysis of the cell. The site of blockage of eculizumab is demonstrated.

in the placebo cohort. Transfusion independence was achieved in 51% of patients in the eculizumab group (22 of 43) and 0% of those in the placebo group (0 of 44, $P < 0{\cdot}001$) (Fig 3). The overall mean rate of transfusion was reduced by 73% in the eculizumab group. Even among patients receiving eculizumab in whom transfusion independence was not reached, the number of units of packed red cells transfused was reduced by 44%, when compared with patients in the placebo group ($P < 0{\cdot}001$). The median time to the first transfusion was 4 weeks in the placebo group and was not reached in the eculizumab group. Before treatment with eculizumab, the haemoglobin levels were maintained by transfusion. With eculizumab treatment, anaemia was improved as indicated by the increase in endogenous erythrocyte mass, and the stabilisation of haemoglobin levels with a concomitant cessation of or reduction in the number of transfusions.

For most patients with PNH, the quality of life is impaired, and fatigue in these patients has been attributed not only to anaemia but also to excessive intravascular haemolysis and the scavenging of NO by cell-free haemoglobin (Rosse, 2000; Rother *et al*, 2005; Hill *et al*, 2006a,b). In this study, the reduction in intravascular haemolysis with eculizumab, when compared with placebo, was associated with a marked and significant improvement in fatigue, as assessed by scores on both the Functional Assessment of Chronic Illness Therapy (FACIT)-Fatigue instrument and European Organisation for the research and Treatment of Cancer Quality of life Questionnaire (EORTC-QLQ-C30) fatigue scale. These improvements with eculizumab occurred without complete resolution of the anaemia, providing further evidence of the contribution of haemolysis, in contrast to anaemia, to the debilitating fatigue in patients with PNH.

Eculizumab has been very well tolerated with no unexpected adverse reactions reported and in the placebo-controlled TRIUMPH study there were more serious adverse events in

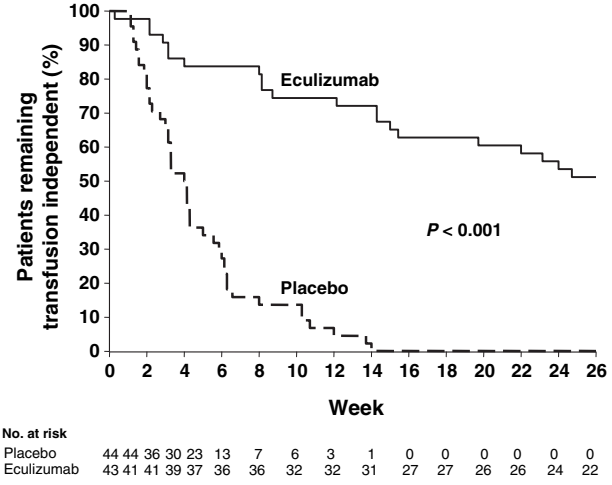

Fig 3. Kaplan–Meier curves for the Time to the First Transfusion during Treatment with Eculizumab. Reprinted with permission from Hillmen *et al* (2006a,b). © 2006 Massacheusetts Medical Society. All rights reserved.

the placebo arm of the trial. There has been no evidence of substantial human antihuman antibody formation in the trials to date. It is expected that there will be an increased risk of *Neisseria* infections, particularly of meningococcal disease. Patients are vaccinated before commencement of eculizumab but the risk will not be completely abolished and therefore vigilance for the symptoms of meningitis or septicaemia is essential. The other theoretical risk is the potential of intense haemolysis if eculizumab treatment is stopped without warning as the number of PNH red cells increases because of their protection from complement-mediated destruction. Patients who elect to discontinue eculizumab treatment should be carefully monitored and possibly transfused with normal red cells depending on clinical circumstances.

Clinical assessment of additional symptoms related to the quality of life in PNH patients, including abdominal pain, dysphagia, and erectile dysfunction, have also been reported to improve during eculizumab therapy (Hill *et al*, 2005b). Many of these symptoms have been attributed to NO depletion by cell-free plasma haemoglobin. Although it is possible that the stabilisation of haemoglobin levels during eculizumab therapy may contribute to the improvement in erectile dysfunction, this mechanism has not been previously observed. Therefore, the improvement in erectile dysfunction for patients on eculizumab is most likely to be due to reduced haemolysis with subsequent reduced NO depletion. There appears to be a tight relationship among complement blockade, haemolysis and symptoms in PNH.

The issue of possible protection against the risk of thrombosis through terminal complement inhibition with eculizumab is being evaluated in the ongoing clinical studies of PNH. The tendency towards thrombosis in patients with PNH may involve high levels of free plasma haemoglobin with the consequent scavenging of NO (Radomski *et al*, 1987a; Sims *et al*, 1989; Wiedmer *et al*, 1993; Olsen *et al*, 1996; Schafer *et al*, 2004; Rother *et al*, 2005), exposure of prothrombotic erythrocyte membranes and/or the absence of GPI-anchored complement inhibitors on the surfaces of circulating platelets (Wiedmer *et al*, 1993; Gralnick *et al*, 1995; Leone *et al*, 2001; Sloand *et al*, 2006). It is reasonable to hypothesise that thrombosis resulting from any or all of these mechanisms should be reduced by terminal complement blockade with eculizumab. Indeed, a preliminary analysis of the rate of thrombosis in the ongoing eculizumab PNH clinical program shows that the thrombosis rate is dramatically reduced when compared with the rate prior to commencement of eculizumab treatment (Hillmen *et al*, 2006b). It is therefore likely that eculizumab will reduce the risk of thrombosis in PNH, which would be expected to have a dramatic impact on the long-term prevention of life-threatening complications in PNH.

Global PNH registry

In view of the infrequency of PNH, it is extremely difficult to perform large or randomised trials of therapeutic interventions.

In addition, the advent of complement inhibitors, such as eculizumab, or the routine use of prophylactic anticoagulation promises to alter the natural history of PNH. In fact, the approval process of eculizumab as a therapeutic agent will be on the basis of relatively small and short clinical trials. Thus, few patients are likely to have received more than 3 years of eculizumab. These factors have led to the recent development of a Global Registry for PNH. It is hoped that all PNH patients will participate in the Global PNH Registry in order that a more thorough insight into the effect of eculizumab on the natural history of PNH can be established and that our understanding of this complex disease will be further enhanced.

Conclusion

The understanding of the biology and natural history of PNH has changed dramatically over the last 10 years. This has facilitated major steps forward in our ability to diagnose and treat the disease. The recent elucidation of the impact of NO depletion on the symptoms and complications associated with haemolysis may lead to further novel approaches to therapeutic options in the future. Recent studies of the terminal complement inhibitor, eculizumab, offer the first promise that we may have a 'targeted' therapy capable of controlling haemolysis in PNH. Eculizumab treatment has demonstrated marked improvements in anaemia, fatigue and quality of life. In addition, initial evidence suggests that eculizumab may be able to reduce the risk of thrombosis, the most feared complication of PNH, and that primary prophylaxis with anticoagulants for selected patients may also be useful. The development of the Global PNH Registry offers the potential to further our understanding of PNH. It appears that the future holds hope for patients suffering from this rare and life-threatening disorder.

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