

Historical Review

HISTORICAL ASPECTS OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA: 'DEFINING THE DISEASE'

This history of paroxysmal nocturnal haemoglobinuria (PNH) is divided into two parts. Part I, the Early History, begins with a review of the initial description of the disease (published in the latter part of the 19th century) and concludes with a recounting of the discovery of the alternative pathway of complement by Pillemer in the early 1950s. Part II, the Modern History, begins with a review of the studies of Dacie and Rosse. The results of their experiments, published in the mid-1960s, defined many of the unique features of the disease. This chronicle ends with a summary of the studies that built on the seminal discovery in 1993 by Kinoshita and colleagues of the genetic basis of PNH.

PART I. THE EARLY HISTORY

Why is it that such a rare disease has so captured the imagination of haematologists that it would be chosen as a topic for a historical review in this venerable journal? Although doubtfully their final lament, I am certain that many astute clinicians go to their grave having never diagnosed a case of paroxysmal nocturnal haemoglobinuria (PNH). Yet PNH is a regular lecture topic at the annual meeting of the American Society of Hematology, and the auditorium in which the lecture is presented is invariably filled with bright-eyed clinicians and scientists, senior and junior, eagerly awaiting a discussion of the most recent developments in the field.

I believe that the enduring fascination with PNH results from the convergence of three factors: (1) the rarity of the disease and its protean clinical manifestations that make diagnosis challenging but particularly gratifying; (2) the intricacy of the pathophysiology; (3) the captivating way in which the fundamental abnormalities have been elucidated systematically over many years (Table I). The elegant complexity of the disease gives it a natural beauty that has stimulated the imagination of biomedical researchers for over a century, and those who have sought to understand the disease have been rewarded often with remarkable new insights (Table I).

Episodic haemoglobinuria is the *sine qua non* of PNH. The intricacy of the pathophysiology of PNH is illustrated by the seven distinct layers of investigation that were required to understand the fundamental basis of this clinically defining

symptom. First, the haemoglobinuria was found to be a consequence of intravascular haemolysis. Second, the intravascular haemolysis was shown to be due to an abnormality of the PNH red cell that resulted in greater sensitivity to lysis by a serum factor. Third, complement was found to be the serum factor that mediates the greater lysis of PNH red cells. Fourth, this greater susceptibility was discovered to be due to aberrant regulation at two distinct sites of the complement cascade. Fifth, deficiency of two membrane-associated inhibitors was shown to underlie the greater complement sensitivity of PNH erythrocytes. Sixth, the complement regulatory proteins were found to share a common post-translational modification [the glycosyl phosphatidylinositol (GPI) anchor]. Seventh, a gene that is required for synthesis of the GPI-anchor (*PIG-A*) was found to be mutant in PNH. Each of these layers will be reviewed in some detail within this narrative. This history illustrates vividly how the study of PNH has rewarded persistence and vision (and how serendipity is involved in many remarkable discoveries).

These discoveries were made steadily over a period of 111 years (1882–1993), and each generation of physicians and scientists during this period made important contributions to the field. The mysteries of PNH have been solved in a particularly satisfying way because the precision and orderliness of the solutions made clearly understandable what had seemed at the time, prior to resolution, to be a problem of nearly insurmountable complexity. Although it isn't true that more people study PNH than have it, it may be true that more people are interested in the disease than have it. If so, it is because learning about PNH is an inspirational reminder of the elegant complexity of nature, the rewards of curiosity, and the power and beauty of science.

Overview

Although some question the diagnostic importance of nocturnal haemoglobinuria [Dacie and Lewis (1972) reported it to be the presenting symptom in only 26% of patients], it was this symptom that defined PNH as a distinct clinical entity and ignited the curiosity of early investigators (Table I). Further, the nocturnal aspect of the paroxysms suggested to Strübing (1882) a mechanism for the haemoglobinuria. He hypothesized that this symptom was a consequence of the abnormal sensitivity of PNH erythrocytes to systemic acidosis resulting from accumulation of CO₂ during sleep. Although the validity is still debated, systematic investigation of this hypothesis by Strübing (1882), Hijmans van den Berg (1911) and Ham (1937) lead

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Table I. PNH – more than an haemolytic anaemia.*

Characteristic (date of discovery or seminal observation when identifiable)

Haemoglobinuria (1866, 1882)
 A consequence of intravascular haemolysis (1882)
 Due to greater sensitivity of the erythrocytes to complement mediated lysis (1939, 1955, 1966)
 Due to absence of complement regulatory proteins (1983, 1989)
 Deficient complement regulatory proteins are GPI-anchored (1986)
 Deficiency of GPI-anchored proteins due to mutation in *PIG-A* (1993)
 Phenotypic mosaicism first defined by differences in complement sensitivity of RBC (1963, 1966)
 Lead to somatic mutation hypothesis (1963)
 Phenotypic mosaicism due to genotypic mosaicism (1996)

Nocturnal paroxysms (1866, 1882)
 Defined PNH as a distinct entity (1882)
 Essentially all of the early research on PNH focused on this symptom (1882–1940)
 Lead to discovery of acidified serum lysis test of Ham (1939)
 Helped confirm the existence of the alternative pathway of complement (1954, 1955)

Thrombophilia (1950)
 Primary cause of morbidity and mortality
 Aetiology uncertain†

Haematopoietic stem cell disease – WBC and platelets share phenotypic and genetic defects with erythroid element (1969)

Bone marrow failure syndrome (1961)
 Close association with acquired aplastic anaemia (1967)
 Lead to natural selection hypothesis to explain the clonal dominance of mutant stem cells (1963)

Clonal disease (1963, 1970)
 But not malignant
 Basis of clonal selection and dominance currently unknown†

Deficiency of multiple cell surface proteins on haematopoietic elements (1951, 1959, 1986)
 Deficient proteins are GPI-anchored (1986)
 Mutant gene (*PIG-A*) required for synthesis of GPI-anchor moiety (1993)
 Phenotypic mosaicism due to genotypic mosaicism (1996)
PIG-A is X-linked (1993)
 Males and females are affected equally because mutations occurs in a somatic tissue (the haematopoietic stem cell) in which only one X chromosome is active in females
 All cases of PNH due to *PIG-A* mutation probably because other genes involved in GPI-anchor synthesis are autosomal (1993–present)

*PNH has fascinated physicians and scientists for more than 100 years because of its elegant complexity. Defining the fundamental basis of the characteristic signs and symptoms of the disease has led to a number of remarkable discoveries.

†Determining the basis of the clonal selection and dominance and of the thrombophilia remain challenges for the future.

directly to the development of a specific diagnostic test for PNH (the acidified serum lysis test of Ham) and to the discovery by Ham of the fundamental role of complement in the lysis of PNH erythrocytes (Ham, 1939; Ham & Dingle, 1939).

In a 1953 review, Crosby reported the high incidence of thrombosis-related deaths in PNH, and subsequent clinical studies have confirmed that thromboembolic events are a major cause of morbidity and mortality (Crosby, 1953a). Thus, in addition to being classified as a haemolytic anaemia, PNH is included on the list of thrombophilic conditions. The fundamental basis of the thrombophilia of PNH is undefined and represents one of two major unsolved mysteries of PNH (Table I) (Dacie, 1963).

PNH is also included among the bone marrow failure syndromes because, at some point during the course of their illness, almost all patients have (in addition to anaemia) thrombocytopenia, leucopenia or both. Further, there is a clear, albeit incompletely understood, connection between

PNH and acquired aplastic anaemia. PNH is also a stem cell disorder because platelets and leucocytes share, with erythrocytes, the deficiency of GPI-anchored proteins (the exact stage of differentiation in which the genetic mutation occurs is undefined, but it must be effected in a very primitive stem cell because mutant *PIG-A* is found in erythroid, myeloid and lymphoid elements from the same patient).

PNH is remarkable because it is a clonal disease but not a malignant disease, and the bone marrow and peripheral blood are mosaics of normal and abnormal cells. Further, individual patients often have multiple abnormal clones that are phenotypically and genotypically discrete. The abnormal stem cells are a consequence of somatic mutation and the mutant gene is located on the X-chromosome. Thus, inactivation of only one gene in somatic tissues is necessary for manifestation of the phenotype, explaining why all cases of PNH are due to mutant *PIG-A* (this hypothesis assumes that all other genes that could cause the

phenotype are autosomal). Genetically, females and males are equally susceptible because only one X-chromosome is active in the somatic tissue of females.

The mutant gene, *PIG-A*, is an essential component of the pathway required for synthesis of the GPI moiety that serves as an anchor for a functionally diverse group of membrane proteins. Of the 20 or so proteins that are deficient on the haematopoietic cells of PNH, only DAF (CD55) and MIRL (CD59) share an obvious functional relationship (they are both complement regulatory proteins). Absence of these two proteins accounts for the marked susceptibility of PNH erythrocytes to complement-mediated lysis. The *PIG-A* mutation is necessary for the development of PNH, but it appears insufficient to result in clonal expansion in the absence of some other selective pressure. Defining the nature of the selective process that results in the expansion and clonal dominance of the *PIG-A* mutant stem cells is currently the most active area of investigation and represents Dacie's ultimate problem – 'the aetiology of the disease and its relationship to marrow hypoplasia' (Dacie, 1963). Whatever the solution to this problem, it must somehow depend on the absence of one or more GPI-anchored proteins.

After reviewing Table I, I hope it is apparent why this elegant, complex disease is a source of continuing fascination for haematologists. This history of PNH is intended to chronicle the landmark events that defined the disease over the past century.

Early history

Both Crosby (1951) and Rosse (2000) have undertaken scholarly studies of the early history of PNH. Although it is likely that descriptions of the disease were published by others [including a noteworthy report by the renown British physician, William Gull (1866)], Paul Strübing of Greifswald, Germany, clearly recognized PNH as an entity distinct from both paroxysmal cold haemoglobinuria and march haemoglobinuria, two similar syndromes also described in the 19th century. Published in 1882, Strübing's paper is a remarkable example of how insightful clinical observations can suggest disease mechanisms and thereby provide direction for laboratory investigation.

A junior physician at the time, Strübing studied a 29-year-old-cartwright beginning in 1880. Based upon the observation that after severe attacks the plasma was red, Strübing concluded that the blood cells were dissolved within the vessels and not in either the kidney or the urine. The astuteness of Strübing's observation is underscored by the fact that, as late as 1911, Marchiafava believed that the haemolysis occurred in the kidney (Marchiafava & Nazari, 1911). [Marchiafava–Micheli disease was an eponym for PNH that was popular during the 1930s and 1940s (Crosby, 1951; Dacie, 1963). Although the eponym lingered into the 1960s, rightfully it has not endured because the contributions of Marchiafava and Micheli were too modest to warrant such recognition. The Dutch physician, Ennekin has been credited with first using the term 'paroxysmal nocturnal haemoglobinuria' in 1928 (Crosby, 1951; Dacie, 1963; Rosse, 2000)].

Strübing (1882) used spectroscopy to demonstrate that the discolouration of the urine was due to the presence of haemoglobin. He also described the characteristic morphological features of the urine sediment of PNH, although he did not recognize that haemosiderin accounted for the pigmentation of the cellular material. Further, he noted that erythrocytes were absent from the urine except after a very severe attack, when a rare red cell was observed. These findings are relevant today, as both haemosiderinuria and haemoglobinuria in the absence of haematuria remain important components of the clinical criteria used to diagnose PNH.

Strübing concluded that sleep played a critically important role in the haemolytic process because only by awakening the patient during the night was the haemoglobinuria observed at a time other than the first voided urine of the morning. He also thought that the destruction of the erythrocytes was a slow, gradual process because there were no signs of rapid haemolysis such as chills, fever or flushing. In developing his eerily prescient hypothesis that the red cells of PNH were destroyed during sleep because they were abnormally sensitive to an acid environment, Strübing drew upon available information that normal red cells were susceptible to lysis *in vitro* if the cells suspension is acidified using carbon dioxide. He reasoned that, during sleep, carbon dioxide and lactic acid accumulated because of slowing of the circulation. According to Strübing's hypothesis, normal erythrocytes were resistant to lysis under these conditions, but the defective PNH cells were vulnerable to the mildly acidic conditions that occurred during sleep. Conceivably, Leonhard Landois, the Professor of Physiology at Greifswald and a colleague of Strübing, contributed to this remarkably insightful hypothesis (Rosse, 2000).

Strübing attempted to support his hypothesis with experimental data by giving the patient acid, but this treatment failed to induce haemoglobinuria. Fifty-five years later, Ham (1937) repeated this experiment by giving patients a single dose of ammonium chloride to acidify the serum *in vivo*. Under these conditions, haemoglobinaemia and haemoglobinuria increased. Perhaps the results of Strübing's experiment were negative because he failed to give an amount of acid sufficient to lower the plasma pH into the range in which complement activation is enhanced.

Apparently, Strübing did not maintain a long-standing interest in diseases associated with haemoglobinuria as he became Director (in 1889) of the Nose and Throat Clinic at the University of Greifswald (Crosby, 1951). Perhaps this career shift contributed to the dearth of recognition that he received for his seminal contributions to the characterization of PNH. There is evidence, however, that his observations were not completely ignored by other investigators.

In 1911, the Dutch physician, Hijmans van den Berg, built upon Strübing's remarkably astute report. Hijmans van den Berg (1911) showed that the red cells of PNH haemolysed *in vitro* in an atmosphere containing carbon dioxide when the cells were suspended in serum from the patient or from either of two normal subjects. He also demonstrated that red cells from normal volunteers did not

lyse under the same experimental conditions. These studies are seminal because they demonstrate conclusively that the haemolysis of PNH is due to a defect in the red cell rather than to the presence of an abnormal plasma factor (as is the case with paroxysmal cold haemoglobinuria).

PNH and complement

A review of the early history of complement is needed to understand why Hijmans van den Berg failed to identify the serum factor required for the lysis of PNH erythrocytes. Jules Bordet is credited with performing the critical experiments that identified complement in 1894 (Ross, 1986). Investigating the killing of *vibrio cholera* by immune serum, he demonstrated that the activity was dependent upon both a heat-stable factor (that we now know is antibody) present only in immune serum and a heat-labile cytotoxic factor (that we now know is complement) present both in normal (non-immune) and immune serum. He also observed that the cytotoxic effects that were lost as a result of heat inactivation could be restored by the addition of a small amount of fresh normal serum that by itself had no bactericidal activity. In 1899, Paul Ehrlich proposed a scheme of humoral immunity in which he used the term complement for the heat-labile cytotoxic factor of serum, because this factor complemented the activity of the heat-stable immune factor. Thus, at the time of Hijmans van den Berg's study, the following two characteristics of complement were accepted: (1) the activity was lost if the serum were heat inactivated; (2) the activity could be restored by adding a small amount of fresh serum.

Consistent with a role for complement in the lysis of PNH erythrocytes, Hijmans van den Berg (1911) reported that haemolysis was no longer observed when the serum source was incubated at 50°C for 30 min. Hijmans van den Berg, however, reached the erroneous conclusion that complement did not mediate the haemolysis of PNH cells *in vitro* because the haemolytic activity of the heated serum was not restored by the addition of a small amount of fresh human or guinea pig serum. He concluded that haemolysis was caused not by a specific haemolytic substances in the serum but by an abnormal fragility of the erythrocytes to carbon dioxide.

Only upon the discovery of the alternative pathway of complement by Pillemer 43 years later (Pillemer *et al.*, 1954) would the reason that the fresh serum failed to restore the haemolytic activity of heat-inactivated serum become apparent. A characteristic feature of processes mediated by the alternative pathway is that the lytic event is no longer observed following modest dilutions of serum (e.g. 1:4 or 1:8). This observation contrasts sharply with classic pathway-mediated lysis that is sustained despite using serum (as the complement source) at relatively high dilutions (commonly > 1:100). This difference explains the observations of early investigators (including Hijmans van den Berg) who dismissed complement as the mediator of lysis of PNH erythrocytes in acidified serum because haemolysis was not observed following dilutions of serum that were known to support antibody-initiated (classic pathway) lysis.

The acidified serum test of Ham

In 1937, Thomas Hale Ham (Fig 1) reported findings that were remarkably similar to the studies of Strübing. There are no references to Strübing in that paper (Ham, 1937), however, indicating that Ham (like most others) was unaware of Strübing's work. As was Strübing before him, Ham was struck by the relationship between the haemolysis and sleep. This relationship lead him to postulate (just as Strübing had done in 1882) that 'Because of the elevation in the carbon-dioxide content of the arterial blood and the decrease in pH known to occur during sleep, it was suspected that a change in acid-base equilibrium was related to the increased haemoglobinaemia of the patients during sleep'.

Ham challenged his hypothesis that acidification of the plasma induced the haemolysis by giving two of the three study patients sodium bicarbonate. He reported that this treatment caused the haemoglobinaemia and haemoglobinuria to decrease. In a corollary experiment, Ham also reported that, following administration of ammonium chloride (to acidify the plasma), the haemoglobinaemia and haemoglobinuria increased (as noted above, Strübing undertook similar experiments. The results of those experiments were negative, however, perhaps because of differences in experimental design).

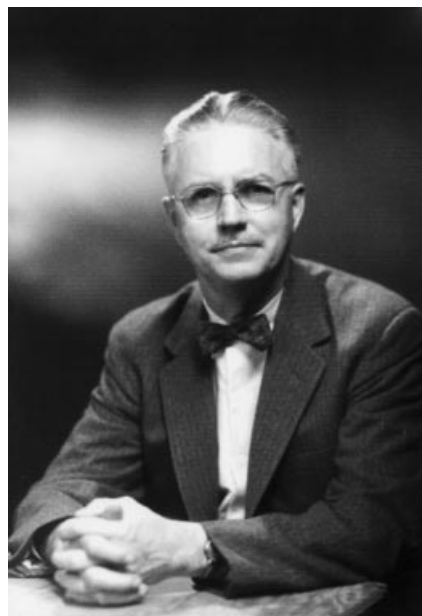


Fig 1. Thomas Hale Ham (1905–87). In 1939, Ham presented evidence that complement mediates the abnormal lysis of PNH erythrocytes (Ham, 1939; Ham & Dingle, 1939). Ham also developed a highly specific diagnostic test for PNH (the acidified serum lysis test). Dr Ham was on the faculty at Case Western Reserve University (Cleveland, OH, USA) at the same time as Louis Pillemer who discovered the alternative pathway of complement in the early to mid 1950s (this photograph of Dr Ham was obtained from the University Archives of Case Western Reserve University. The owner of the copyright could not be identified).

As further evidence of the role of acidification in the haemolysis of PNH, Ham noted that, in one of his study patients, the arterial pH was 7.3 during 'natural sleep' and during that time the 'usual haemoglobinemia and haemoglobinuria occurred' (Ham, 1937). When the patient was subjected to hyperventilation using a Drinker artificial respirator, the arterial blood pH was observed to be 7.47 in association with a pCO₂ of 28 mm. Under these conditions a decrease in plasma and urine haemoglobin was noted.

Armed with the compelling results of the *in vivo* studies, Ham sought to investigate his hypothesis using *in vitro* techniques. Although his experimental design was remarkably similar to that of Hijmans van den Berg, it appears that Ham was not familiar with the Dutch physician's work on PNH [Hijmans van den Berg is not referenced in Ham's 1937 study (although his work is cited in Ham's two papers of 1939 (Ham, 1939; Ham & Dingle, 1939))]. Ham made the following observations about the erythrocytes from the three study patients: (1) rapid haemolysis was observed when the serum or plasma was acidified by using either equilibration with CO₂ or addition of lactic acid; (2) the effects of CO₂ were inhibited by the addition of sodium bicarbonate; (3) the haemolysis was observed if serum or plasma from normal volunteers was substituted for patients' serum or plasma; (4) blood Group O red cells from normal volunteers were not haemolysed when resuspended in patients' serum or plasma that was subsequently acidified with CO₂ or lactic acid. Based on these observations, Ham reached the following conclusion: 'Thus the essential peculiarity in these patients apparently resides in the red blood cells, whereas a factor essential for haemolysis is common to the plasmas or serums of the patients and of all normal subjects investigated' (Ham, 1937). This statement accurately and concisely summarizes the characteristics of the haemolysis of PNH, and the conclusions closely resemble those of Hijmans van den Berg (1911).

As had Hijmans van den Berg 26 years earlier, Ham studied the role of complement in the lytic process. He observed that the haemolysis did not occur if the serum or plasma were heated for 30 min at 50°C or 60°C and, like Hijmans van den Berg, Ham found that the haemolytic activity was not restored by the addition of complement in the form of fresh human serum (20% v/v, a 1:5 dilution) or by a small amount of guinea pig serum (Ham, 1937). He also observed that the haemolytic activity was inhibited by adding sodium citrate, potassium oxalate or potassium cyanide to the plasma or serum. All these salts were known inhibitors of complement-mediated lytic systems.

Ham correctly concluded that a thermostable factor was essential for the haemolysis that was observed when PNH erythrocytes were incubated in acidified serum or plasma (Ham, 1937). He went on to note five reasons why the haemolytic system that defined PNH differed from that of paroxysmal cold haemoglobinuria owing to the Donath-Landsteiner antibody. His fifth reason was that 'since the addition of complement did not reactivate the heated serums, the haemolysis described above did not depend upon an antigen-amboceptor-complement system' (Ham

used Ehrlich's 1899 terminology to describe the elements of humoral immunity in which amboceptor receptor represents what we now know to be immunoglobulin). Thus, Ham recognized that the mechanism by which PNH erythrocytes are lysed in acidified serum differed from that of known systems. However, as Hijmans van den Berg before him, Ham arrived at the erroneous conclusion that the haemolysis was not mediated by complement because standard reconstitution experiments using dilutions of fresh serum gave negative results.

In 1939, Ham and Dingle published a landmark paper that influenced the course of PNH research for the next 50 years. The subtitle of the paper is 'Certain Immunological Aspects of the Hemolytic Mechanisms with Special Reference to Serum Complement.' The paper, a model of thoughtful, rigorous investigation, suggested a novel mechanism by which the abnormal erythrocytes of PNH were lysed by an immune mechanism independent of antibody.

Building on previous observations that the haemolysis of PNH cells was inhibited by heat inactivation of serum or by the addition of certain salts that were known to inhibit complement (Ham, 1937), Ham and Dingle investigated each facet of the humoral immune system (antigen, antibody and complement) as it was known to them at the time. They divided their report into seven sections (Ham & Dingle, 1939). Although the paper is cited primarily because of the detailed analysis of the role of complement in the lysis of PNH erythrocytes, only section 2 was concerned with this relationship. Equally important were the two sections that dealt with the investigation of the role of antibody in the lytic process. That no evidence of antibody was found either in patient serum (section 1) or associated with patient red cells (section 4) implied the existence of a novel pathway of immune lysis.

The authors also demonstrated that PNH red cells were more susceptible to lysis than normal red cells when incubated with antibody [either rabbit anti-human red blood cell (RBC) antiserum or iso-haemolysins] and human serum (section 6) (Ham & Dingle, 1939). Lysis of PNH and normal red cells did not differ, however, in non-immunological systems (saponin, sodium taurocholate and hypotonic sodium chloride) (section 7). The antigenic properties of the red cells were examined in section 3 by injecting PNH and normal erythrocytes into rabbits and examining the characteristics of the antisera that were generated. No differences were observed, but clearly the technique was too crude to detect the relatively subtle differences that we now know exist (i.e. absence of GPI-anchored proteins). Further, only ~15% of the PNH red cells used in this experiment were susceptible to acidified serum lysis. As erythrocytes from patients with PNH are a mosaic of normal and abnormal cells, the relatively low percentage of susceptible cells implies that the majority of the cells from the PNH patient that were used for immunization lacked the disease phenotype.

In section 5, the authors demonstrated that the characteristics of lysis of PNH red cells in acidified serum were markedly different from those of lysis of normal cells using

isohaemolysins (i.e. anti-blood group A and anti-blood group B antibodies); again, consistent with a novel mechanism of haemolysis different from known antibody-initiated processes.

Thus, in addition to their seminal observations on the role of complement in the lysis of PNH red cells, Ham and Dingle (1939) made several other critically important discoveries about the nature of the lysis of PNH cells in acidified serum. Their finding that antibody did not participate in the lytic process was particularly remarkable because such a process was unprecedented. The rigour with which the authors approached the investigation of the role of antibody in the lysis of PNH cells by acidified serum suggests that they realized the uniqueness of their observation and the scepticism that it might evoke.

The authors were equally rigorous in their investigation of the role of complement in acidified serum lysis. They used available methods to increase complement concentration, decrease complement concentration, inhibit complement, and remove or inactivate fractions or components of complement (Ham & Dingle, 1939). The bulk of the data suggested that the lytic substance of serum was complement; however, inconsistencies in the results persuaded Ham and Dingle to be conservative (and in some instances contradictory) in their interpretation of the experiments. At one point they concluded the following: 'Although serum complement cannot be identified directly, it appeared from this evidence that the serum factor in this haemolytic mechanism corresponded in its general behaviour to complement or to a complement-like substance. It did not appear likely, however, that complement itself was the haemolytic agent.' At another point, they surmise that 'The haemolytic system involved cannot be classified strictly as immunological in nature since there has been no demonstration of antigen or antibody. It is probable, however, that the haemolytic mechanism is an immunological system since complement or a complement-like substance is required for haemolysis and, presumably, because the red cells themselves show increased susceptibility to haemolysis in certain known immunological haemolytic systems.' The latter part of this statement is based on their observation that PNH erythrocytes were more susceptible to antibody-initiated lysis when human serum was used as the source of the lytic substance. This finding was subsequently exploited by others to characterize the unique phenotypic mosaicism of the erythrocytes of PNH (Dacie, 1949; Rosse & Dacie, 1966). The final conclusion of Ham and Dingle that 'The serum factor essential for haemolysis was closely associated with, if not indistinguishable from complement or alexin of human serum' suggests that the authors believed that the lytic substance of serum was indeed complement (alexin is Buchner's term for complement introduced in 1889). The deviation of some of the results from the accepted properties of complement-mediated lytic systems, however, appears to have persuaded them to interpret their findings cautiously.

Relationship of haemoglobinaemia and haemoglobinuria to sleep
At this point, a comment on the relationship between sleep and haemoglobinuria seems appropriate, as it was this

clinical feature that fascinated early investigators and distinguished PNH from other haemolytic anaemias (Table I). The symptom is also important to patients because it is a disconcerting reminder of the chronic nature of their illness and often serves as a dreaded harbinger of an exacerbation of the disease. Finally, general audiences remain enamoured with this symptom (undoubtedly in large part because the name of the disease focuses attention on this peculiar feature). Thus, following any lecture on PNH, a question that invariably arises is 'Why does the haemolysis occur at night?'

Ham stated his hypothesis about the relationship between haemoglobinaemia and sleep as follows: 'Sleep is known to be associated with decreased pulmonary ventilation, with slight elevation of the carbon dioxide content of the arterial blood with a consequent decrease in pH... For this reason it was suspected that elevation of the haemoglobin content of the plasma during sleep was associated with variation in the acid-base equilibrium of the blood' (Ham, 1939). Ham investigated this hypothesis using his usual rigorous approach.

He first demonstrated that haemoglobinuria markedly increased during sleep. In this experiment, the daily excretion of haemoglobin was quantified and divided into two time periods [9 a.m. to 9 p.m. (period of waking) and 9 p.m. to 9 a.m. (period of sleep, although the patient actually slept from 9 p.m. to 6 a.m.)] (perhaps there were fewer distractions on the ward of the Clinical Research Center in Ham's day, making a 9 p.m. bedtime feasible). The analysis of the daily excretion of haemoglobin was performed for 10 consecutive days, and the results clearly showed a marked increase in haemoglobinuria during the sleep period.

Next, Ham investigated the relationship between sleep and haemoglobinaemia (Ham, 1939). First, he showed that, when the patient was kept awake for 27 consecutive hours, the normal pattern of increased nocturnal haemoglobinaemia was no longer observed. He then altered the pattern of sleep so that the patient slept during the day and was awake at night. Under these conditions, haemoglobinaemia was once again observed to increase during the sleep period. On the other hand, bed rest without sleep did not result in an increase in haemoglobinaemia. Thus, paroxysmal nocturnal haemoglobinuria is something of a misnomer, as increased haemolysis is related to sleep, rather than to time of day.

To challenge his hypothesis that the hypoventilation associated with sleep is the critical factor that is ultimately responsible for the increase in haemoglobinaemia during sleep, Ham used a Drinker respirator to control artificially both the respiratory rate and the respiratory volume of a patient (Ham, 1939). He observed that hyperventilation during 6 h of sleep produced alkalosis and was accompanied by no increase in either haemoglobinaemia or haemoglobinuria. During sleep without hyperventilation, a more acid pH was observed, accompanied by an increase in both haemoglobinaemia and haemoglobinuria.

In 1953, Crosby challenged Ham's findings (Crosby, 1953a). Also using a Drinker respirator, Crosby reported (as

an documented observation) that the pattern of nocturnal haemoglobinuria was not reversed when the speed and amplitude of the respiratory cycle of a patient with PNH was set so that the parameters were greater than those of the patient's normal waking state. This experiment was designed to insure that CO₂ did not accumulate as a consequence of relative hypoventilation during sleep. The patient was observed for 10 consecutive nights, and although the pattern of nocturnal haemoglobinuria was unaltered, Crosby concluded that 'The "acid shift" undoubtedly contributes to the 'nocturnal' nature of the disease, but it is not the only pathological mechanism' (Crosby, 1953a). Others have also questioned whether acidification of plasma is an important factor in the relationship between sleep and haemoglobinaemia. Further, not all patients with PNH are observed to have an increase in haemoglobinaemia during sleep.

Conceivably, among patients who have a nocturnal increase in haemoglobinaemia, the blood may be acidic in localized areas where the rate of flow is slowed during sleep, but the pH of arterial blood sampled from an extremity may not reflect such heterogeneity. In support of this hypothesis, Strübing (1882) noted that the night subsequent to heavy exercise or overwork produced an attack of haemoglobinuria. He thought that accumulation of lactic acid from the previous day's exertion contributed to the acid environment needed to induce haemolysis. Blum *et al* (1967) provided experimental support for Strübing's hypothesis 85 years later when they reported that the exacerbation of haemolysis noted after strenuous exercise is associated with an increase in plasma lactic acid concentration and a concordant reduction in the pH of the blood.

The basis of the increase in haemoglobinaemia and haemoglobinuria that occurs during sleep is not an area of active investigation. Thus, it is unlikely that new information that addresses this issue will be forthcoming (although serendipity could intervene). Based on available evidence, it is impossible to conclude unequivocally that the nocturnal aspect of the haemoglobinuria is due to acidification of the plasma (and consequent activation of complement) resulting from sleep-associated hypoventilation and CO₂ accumulation. The question of the relationship between haemolysis and sleep is interesting from a historical perspective, and there would be satisfaction in being able to respond decisively to those who ask why the haemolysis occurs at night. More importantly, a better understanding of the relationship between sleep and haemolysis might suggest more specific therapy for this troubling symptom. Although investigation into the basis of the nocturnal nature of the haemolysis is currently dormant, the role that this peculiar manifestation of the disease had in the unravelling of the fundamental mechanism that underlies PNH cannot be overemphasized.

Discovery of the alternative pathway of complement

The decade that followed the publication of the landmark studies of Ham & Dingle in 1939 produced limited progress toward defining the basis of the abnormal susceptibility of PNH erythrocytes to haemolysis in acidified serum. As had

Ham and Dingle (1939), others found evidence for involvement of complement, but deviations from the accepted characteristics of a complement-mediated lytic system were consistently observed as well. After reviewing the work of Ham and Dingle, Dacie (1949) made the following statement: 'There is thus some evidence for and some evidence against the participation of human serum complement in the haemolysis by serum of erythrocytes of patients with nocturnal haemoglobinuria and in the present state of knowledge any theory of the mechanism of haemolysis can only be speculative'.

Even the role of divalent cations in the *in vitro* haemolytic process associated with PNH was debated into the mid-1950s (Crosby, 1953b). Harris *et al* (1951) observed that the process responsible for the lysis of PNH erythrocytes *in vitro* differed from haemolytic complement by its dependence on magnesium and its inhibition by calcium (as an interesting historical aside, Louis Pillemer was a co-author on the abstract that reported those observations, and the submission was introduced to the American Society of Clinical Investigation by Thomas Hale Ham). As an isolated event, the findings of Harris *et al* (1951) appear to represent only a minor incremental advance. In retrospect, however, the observation that the haemolysis of PNH was a magnesium-dependent phenomenon can be seen as a pivotal.

Pillemer *et al* (1954) reported the isolation of properdin. The protein was discovered serendipitously while Pillemer and colleagues were attempting to isolate one of the components of C'3 (at that time, the 'third' component of complement, designated C'3, actually consisted of several discrete proteins that co-purified using available techniques). Properdin was isolated by eluting it from zymosan that had been incubated with serum (zymosan is the insoluble residue from yeast that has been digested with trypsin and extracted with water and alcohol). Pillemer *et al* (1954) noted that properdin acted 'only in conjunction with complement and magnesium and participates in such diverse activities as the destruction of bacteria, the neutralization of viruses, and the lysis of certain red cells.' The certain red cells that Pillemer was referring to were from patients with PNH. A year earlier (Pillemer *et al*, 1953a,b), he noted the striking resemblance between the mechanism of lysis of PNH erythrocytes and the inactivation of complement by zymosan (both reactions required magnesium and serum components resembling complement and both proceeded optimally at pH 7, but neither system required a specific antibody). Pillemer *et al* (1954) described briefly the results of experiments performed with Hinz and Jordan that indicated that the properdin system was involved in the lysis of PNH erythrocytes in acidified serum. A paper reporting those experiments in detail was published a few years later (Hinz *et al*, 1956), which showed that serum depleted of properdin (RP) had no haemolytic activity for PNH erythrocytes in acidified serum, whereas RP was fully active in mediating lysis of red cells in antibody-dependent systems (Table II). Repleting the RP with isolated properdin restored its capacity to support lysis of PNH red cells in acidified serum (Table II). The simple but elegant studies described in this paper strongly supported Pillemer's

Table II. The relationship between serum properdin levels and haemolysis of PNH erythrocytes.*

Serum	Properdin (units/ml)	Haemolysis of PNH (%)
Normal – No. 1	4–8	33
Normal – No. 2	1–2	14
RP	0	0
RP + 3 units properdin	3	23
RP + 5 units properdin	5	36

*Acidified serum lysis of PNH erythrocytes is mediated by the alternative pathway of complement. The results demonstrate that properdin is required for the lysis of PNH erythrocytes in acidified serum. A concentration-dependent effect is also shown by these experiments. RP indicates that the serum has been depleted of properdin. Modified from Hinz *et al* (1956) by copyright permission of the American Society of Clinical Investigation.

hypothesis of an antibody-independent lytic system requiring properdin. Thus, studies into the mechanism of lysis of PNH erythrocytes played an important (but largely under-appreciated) role in defining the existence of the alternative pathway of complement.

Initially, properdin was envisioned as functioning in a manner analogous to antibody (i.e. binding of properdin to the cell surface was thought to initiate activation of complement). However, in contrast to antibody (which is a component of acquired immunity), Pillemer assigned properdin as the central element of the innate or natural immune system (Pillemer *et al*, 1954). This recognition of a mechanism for complement activation that did not require antigenic stimulation was a brilliant conceptual leap strongly supported by rigorous experimental data. According to Pillemer's hypothesis, properdin is present in the serum constitutively (i.e. synthesis of the protein does not depend on an antigenic stimulus), and binding is determined, not by recognition of a specific antigen, but by the innate biochemical properties of the cell surface. Thus, constituents of self (e.g. erythrocytes) remain unharmed because properdin has no natural affinity for host cells. In contrast, the system protects against infections because properdin binds to invading organisms (e.g. bacteria, yeast and viruses) through a recognition system that is determined by the foreign nature of the composition of the surface of the cell membrane. Although the details of the mechanism of activation of the alternative pathway are now known to differ from this model (discrimination of self and non-self is mainly determined by the factor H component of the alternative pathway rather than by properdin), the basic concept suggested by Pillemer is remarkably accurate. The alternative pathway (the current name of the properdin system) is an essential component of innate immunity, and discrimination between self and non-self by the alternative pathway is determined by the surface properties of the cell.

In an uncannily insightful review of PNH published in 1963, Dacie (Fig 2) stated that the conclusion that the



Fig 2. John V. Dacie (1912–). Professor Dacie made many important contributions to the clinical and laboratory description of PNH. Among these contributions were the connection between PNH and aplastic anaemia and that PNH arose by somatic mutation affecting the pluripotent haematopoietic stem cell. Professor Dacie's remarkably clear vision continues to guide PNH research today (this photograph of Professor Dacie at age 87 was used with his permission).

properdin system was responsible for the lysis of PNH erythrocytes in acidified serum 'seems generally to have been widely accepted' (Dacie, 1963). He went on to propose a model that could account for the abnormal sensitivity of PNH erythrocytes. According to Dacie's hypothesis, the surface of PNH red cells is abnormal, and 'it is possible that this leads to the adsorption of the properdin complex, while normal cells by virtue of their normal surface fail to do this to a significant degree. The fixation of the complex to the cell surface then leads enzymatically to haemolysis.' He went on to speculate that 'Presumably it is the complement complex which actually brings about the lysis, with properdin, by fixing the complement to the cell, playing a role analogous to that played by classical amboceptor' (surprisingly, Dacie used the antiquated term, amboceptor, instead of the more modern term, antibody, in this review that was given during his tenure as President of the Pathology Section of the Royal Society). It appears likely that Dacie's hypothesis was built upon Pillemer's view of the properdin system. Although another quarter-century would elapse before the details of the process were finally pieced together, conceptually Dacie's model of the aberrant interactions of complement with the erythrocytes of PNH was entirely correct.

The wide acceptance of the role of the properdin system in the lysis of PNH erythrocytes appears to have been the result of two factors. First, the data from the studies of Pillemer *et al* (1954) and Hinz *et al* (1956) were compelling. Second, the hypothesis fitted precisely with what was known about acidified serum lysis of PNH erythrocytes (magnesium along with components resembling complement were required, but antibody was not involved). Regrettably for Pillemer, doubt existed within the immunological community of the existence of the properdin path-

way. The scepticism was in large part owing to the fact that preparations of purified properdin were found to contain small amounts of natural antibodies to yeast and bacteria (protein purification was in its infancy at the time and isolation to homogeneity was in most cases technically impossible). In 1957, Pillemer died of a barbiturate overdose that was ruled a suicide (Ross, 1986). Apparently, he was depressed following a meeting at which other complement investigators were unwilling to acknowledge the existence of the properdin system. Ten years after his death, he was vindicated when others rediscovered the alternative pathway, and by 1980 all the components of the system had been identified and isolated to homogeneity.

We now know that the role of properdin in the alternative pathway is different from that envisioned by Pillemer *et al* (1954) who thought that the protein that he discovered functioned in a manner analogous to that of antibody in the classic pathway. The alternative pathway does not require an initiating factor, however, and the function of properdin is to stabilize and thereby extend the enzymatic half-life of the amplification C3 (C3bBbP) and C5 (C3bBbC3bP) convertases. Nonetheless, Pillemer's vision of the properdin pathway as the effector of innate immunity proved prescient, as this concept is a central tenet of modern immunology. The discovery that the properdin system mediates lysis of PNH erythrocytes in acidified serum solved a problem that had challenged Hijmans van den Berg, Ham, Crosby, Dacie and others. The role of Pillemer in delineating the basis of the haemolysis of PNH erythrocytes is often overlooked, but his contributions were singular.

PART II. THE MODERN HISTORY OF PNH

Phenotypic mosaicism

In discussing the acidified serum lysis test of Ham, Dacie commented in his 1963 review of PNH that 'Even if the serum is changed several times there appear to be always some cells which resist haemolysis' (Dacie, 1963). As had Ham and Dingle (1939), Dacie (1963) observed that PNH erythrocytes were abnormally sensitive to lysis when complement was activated by antibody but, again, complete haemolysis was never observed. These observations suggested the existence either of one population of cells that varied in sensitivity to lysis or of two populations, one abnormally sensitive to lysis and the other of normal sensitivity.

Based in large part on a biphasic red cell survival pattern for transfused PNH erythrocytes, Dacie (1963) favoured the hypothesis that the erythrocytes of PNH consisted of two populations (the curve showed a steep slope early suggesting a population with a very short life span and a less steep slope which approximated that observed for normal erythrocytes). In the early 1960s, the pioneering work of Manfred Mayer at Johns Hopkins University (Baltimore, MD, USA) made possible quantitative analysis of complement activity. To analyse the complement sensitivity of PNH erythrocytes, Rosse (Fig 3) (working with Dacie at the time) modified the technique that Mayer had developed to assay for serum complement (Rosse & Dacie, 1966). The results of

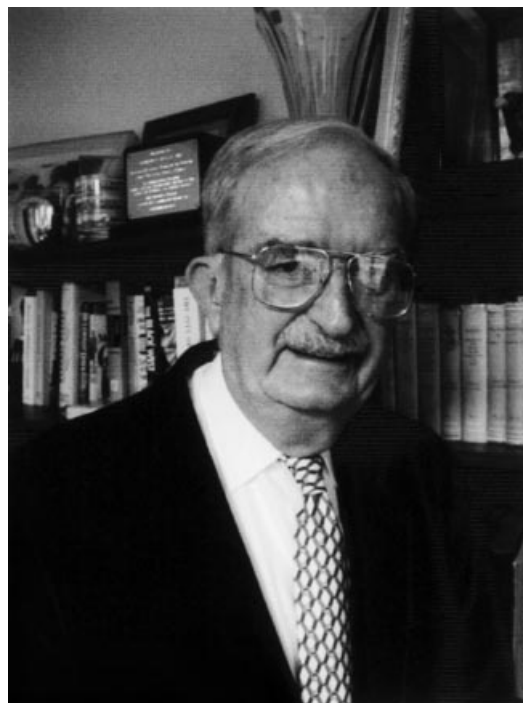


Fig 3. Wendell F. Rosse (1933–). While working with Professor Dacie at Hammersmith in London in 1963 and 1964, Dr Rosse developed the complement lysis sensitivity assay. Using this assay, he and Dacie demonstrated that erythrocytes from patients with PNH are a mosaic of normal and abnormal cells. Dr Rosse also devised experiments that defined the basis of greater sensitivity of PNH erythrocytes to complement. Dr Rosse spent most of his academic career at Duke University in Durham, NC, USA (this photograph, taken by Simone Rosse, was provided by Dr Wendell Rosse and used with his permission).

those studies demonstrated for the first time the magnitude of the difference in sensitivity between PNH and normal erythrocytes and clearly separated the PNH erythrocytes into two quantitatively definable populations (Fig 4).

In the complement lysis sensitivity (CLS) assay of Rosse and Dacie (1966), erythrocytes were incubated with an excess of sensitizing antibody and with incremental (initially limiting) concentrations of serum as the complement source. Subsequently, lysis was quantified based on haemoglobin released. When the results are plotted as the logarithm of the fraction of cells lysed/fraction of cells unlysed versus the logarithm of the complement concentration, a straight line was observed for normal erythrocytes (Fig 4). In contrast, erythrocytes from patients with active PNH showed two connected, nearly parallel straight-line portions of the curve (Fig 4). These findings were interpreted to mean that the peripheral blood of patients with PNH consisted of two populations of cells that differed in susceptibility to complement-mediated lysis. One population (the sensitive population) required ~4% as much serum for an equal degree of lysis as normal cells, while the insensitive population required ~50% as much serum as normal cells for the same degree of haemolysis.

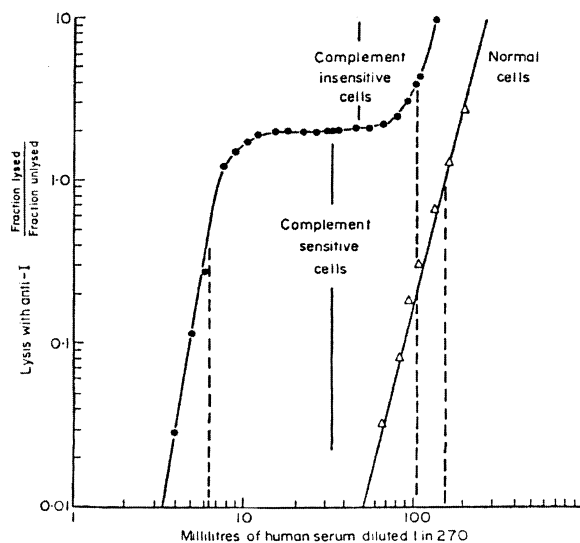


Fig 4. Demonstration that PNH erythrocytes are a mosaic. RBC from a patient with PNH (●) and from a normal volunteer (△) were incubated with antibody and incremental dilutions of serum as the complement source. Subsequently, haemolysis was quantified. The antibody was used in excess and the complement concentration was the limiting factor. The inflection point of the curve that marks the end of lysis of the complement-sensitive cells and the beginning of lysis of the complement-insensitive cells is indicated on the graph by the solid lines. The dashed lines mark the dilution of serum required to produce 50% lysis of normal RBC and of the complement-sensitive and complement-insensitive PNH cells [modified from Rosse (1973) by copyright permission of Blackwell Science Ltd].

The seminal studies of Rosse and Dacie (1966) demonstrated conclusively that the erythrocytes of PNH are a mosaic. However, because the insensitive population was somewhat more susceptible to complement than normal, they concluded that 'PNH cannot simply be regarded as a mosaic of abnormal cells proliferating along with normal cells but, in most cases, as a mosaic of two abnormal populations.' Rosse and Dacie (1966) also interpreted their results as establishing unequivocally that abnormal sensitivity to complement underlies the greater haemolysis of PNH erythrocytes 'regardless of the factors responsible for the initiation of the immune reaction.' This conclusion was strongly supported by the observation that the cells that underwent haemolysis in the acidified serum lysis test (Ham's test) were the same as the sensitive population defined by the complement lysis sensitivity assay (Rosse & Dacie, 1966).

By examining 11 patients, the variation in the proportion of complement-sensitive cells was also demonstrated (the range was from 4% to 80%) (Rosse & Dacie, 1966). This observation provided a plausible explanation for the variability among patients in the severity of the haemolytic component of the disease, thereby illuminating the basis of another remarkable clinical feature of the disease.

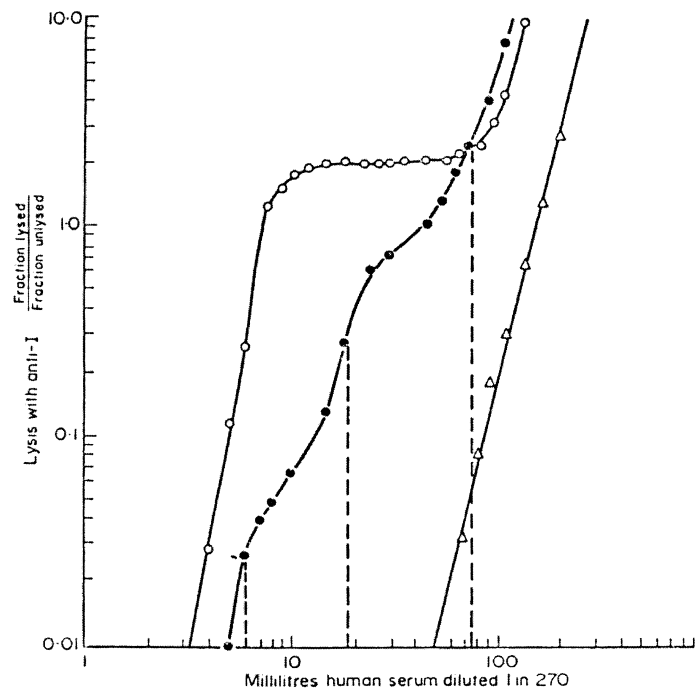
The studies of Rosse and Dacie represented a major conceptual advance and defined many of the fundamental

characteristics of PNH. These studies also greatly influenced future investigation. In particular, the recognition that the basic defect underlying the disease was represented in the complement-sensitive population provided a means for assessing whether a particular observation was PNH specific. According to this paradigm, if a process is specific for PNH erythrocytes, it should be observed in the complement-sensitive population, but to a lesser extent (or not at all) in the insensitive population. For example, by separating cell populations into complement sensitive- and complement-insensitive groups, the importance of the deficiency of erythrocyte acetylcholinesterase in PNH was firmly established (Kunstling & Rosse, 1969). Twenty-five years passed between the discovery of the acetylcholinesterase deficiency in PNH and the time the basis of the deficiency became apparent (Davitz *et al*, 1986). The association between erythrocyte acetylcholinesterase deficiency and PNH, however, lead ultimately to an understanding of the one of the most fundamental mechanisms of the disease (i.e. that the proteins deficient in PNH are GPI-anchored).

Studies indicating that the complement-sensitive erythrocytes were monoclonal while the insensitive cells were polyclonal (Oni *et al*, 1970) provided critically important experimental support for Dacie's hypothesis that somatic mutations account for the phenotypic mosaicism of PNH (Dacie, 1963). Aster and Enright (1969) demonstrated that platelets and neutrophils from PNH patients are abnormally sensitive to complement-mediated lysis. These studies were consistent with the hypotheses that the mutational event occurs in a primitive haematopoietic stem cell (inasmuch as the existence of a pluripotent stem cell was still an issue of active debate at that time, these studies also supported those who hypothesized the existence of a bone marrow cell that could differentiate along erythrocytic, myelocytic and megakaryocytic lines). By using a modification of the complement lysis sensitivity assay, Stern and Rosse (1979) confirmed that, like the erythrocytes, the granulocytes of PNH are also a mosaic.

As noted above, the red cells of patients with PNH were initially divided into two groups (sensitive and insensitive) based on susceptibility to haemolysis by complement (Rosse & Dacie, 1966). According to this classification, the insensitive population was on average twice as sensitive to complement-induced haemolysis as normal erythrocytes. However, with more experience using the assay, it became apparent that the complement-insensitive population was heterogeneous (Rosse, 1973). Careful analysis of the data generated by the complement lysis sensitivity assay showed that three populations of erythrocytes could be identified in some patients with PNH (Fig 5). Based on these observations, Rosse (1973) proposed the phenotypic classification shown in Table II. In the vast majority of patients, a population of cells with complement sensitivity equivalent to that of normal cells was observed along with a variable proportion of abnormally sensitive cells. Erythrocytes of intermediate sensitivity (the PNH II phenotype) could be identified co-existing with cells of normal sensitivity (the PNH I phenotype), marked sensitivity (the PNH III phenotype) or both. The finding of three different red cell

Fig 5. Demonstration that more than one abnormal erythrocyte phenotype can be present in an individual patient. Using the complement lysis sensitivity assay described in Fig 4, Rosse analysed the RBC from two patients with PNH and a normal volunteer. The erythrocytes from the first patient (○) consisted of two populations, complement-sensitive and complement-insensitive. The RBC from the second patient (●) consisted of the following three populations: a markedly sensitive population (PNH type III); a population of intermediate sensitivity (PNH type II); and a population with nearly normal sensitivity (PNH type I) (see Table III). The dilution of serum required to produce 50% lysis of each of the three PNH populations in the second patient is indicated by the dashed line. These studies lead to the hypothesis that PNH does not arise simply by monoclonal expansion [modified from Rosse (1973) by copyright permission of Blackwell Science Ltd].



phenotypes challenged the hypothesis that PNH arose simply by monoclonal expansion (Rosse, 1973). The functional basis of the variability in complement sensitivity of PNH erythrocytes was delineated in 1989 (Holguin *et al.*, 1989a), and in 1996 the molecular basis of the phenotypic mosaicism was defined (Endo *et al.*, 1996).

Functional basis of the abnormal sensitivity of the erythrocytes of PNH to complement-mediated lysis

In a landmark paper, Logue *et al.* (1973) reported that PNH erythrocytes bound more C3 than normal erythrocytes when complement was activated by either the classical or the alternative pathway. They also observed that, for a given amount of C3 bound, PNH erythrocytes lysed to a much greater degree than normal cells. These results suggested that both quantitative and qualitative differences in complement interactions with PNH erythrocytes effected the greater lytic sensitivity. The accurate interpretation of these laborious, technically challenging experiments provided the conceptual framework for subsequent studies aimed at delineating the aberrant interactions of complement with PNH red cells.

Over the next 12 years, the molecular basis of the quantitative and qualitative differences in complement interactions between PNH and normal erythrocytes was defined at the molecular level. A major technical advance that made these studies possible was the development of methods for purifying to homogeneity the components of the complement system. By the early 1980s it was possible to assemble the entire complement cascade using isolated components. A great deal of work by a number of investigators is summarized below.

- The greater C3 binding to PNH II and PNH III erythrocytes is due to aberrant regulation of the C3

convertase of the alternative or classic pathway of complement (Logue *et al.*, 1973; Rosse *et al.*, 1974; Packman *et al.*, 1979; Parker *et al.*, 1982, 1984a, 1985)

- The greater efficiency of lysis of PNH erythrocytes is due to aberrant regulation of the membrane attack complex (MAC) of complement (Rouault *et al.*, 1978; Packman *et al.*, 1979)

- Aberrant regulation of the MAC results in greater C9 binding to PNH erythrocytes compared with normal erythrocytes (Parker *et al.*, 1985)

- Regulation of the MAC is more abnormal on PNH III cells than on PNH II cells (Parker *et al.*, 1985).

Identification of erythrocyte membrane proteins that regulate the C3 convertase

Fearon (1979) reported the isolation of a protein from normal human erythrocytes that inhibited the activity of the C3 convertase of the alternative pathway, and subsequent studies (Fearon, 1980) demonstrated that the inhibitory factor was the membrane receptor for C3 known as complement receptor type I (CR1).

The functional properties of CR1 (CD35) suggested that its absence from erythrocytes could result in greater binding of C3 to cells because such a deficiency would be manifested by enhanced formation and stability of the C3 convertase. Accordingly, experiments designed to characterize CR1 on PNH erythrocytes were performed, but these studies showed that CR1 deficiency did not play a role in the aberrant regulation of complement on PNH erythrocytes (Parker *et al.*, 1984b; Roberts *et al.*, 1985; Ross *et al.*, 1985).

Although CR1 is uninvolved in the pathophysiology of PNH, isolation and characterization of the protein was nonetheless a watershed event because it confirmed the existence of discrete membrane constituents that function

specifically as complement regulators. Further, Fearon's studies demonstrated that membrane-bound complement regulatory factors could be purified to homogeneity while retaining functional activity.

A decade earlier (1969), Hoffmann had published the results of a series of rigorous experiments showing that an extract prepared from human erythrocyte stroma contained a factor or factors that inhibited complement-mediated haemolysis (Hoffmann, 1969a,b). He also showed that a portion of the active material possessed the capacity to bind to the indicator cell and remain functionally active. Subsequently, Hoffmann used the name decay accelerating factor (DAF) to describe the functional property of the extract because the material enhanced the rate at which the activity of the classic pathway C3 convertase diminished over time (Hoffmann *et al*, 1974).

Based on the observations of Hoffmann, Chua *et al* (1980) hypothesized that deficiency of DAF could account for the greater fixation of C3 to PNH erythrocytes. In 1980, those investigators demonstrated that Hoffmann's extract from normal erythrocytes was a potent inhibitor of complement-mediated lysis of PNH erythrocytes. When the DAF activity of erythrocyte extracts derived from PNH and normal cells was compared, however, no difference was observed. Even the extract from a uniform population of PNH III erythrocytes was found to have normal complement lysis inhibitory activity. The reason why no difference in DAF activity was observed is speculative; however, technical factors must have must accounted for the negative results.

One of the complement regulatory proteins contained in the extracts described by Hoffmann was purified to homogeneity by Nicholson-Weller *et al* (1982). This protein was named decay accelerating factor of stroma (DAF-S). The term DAF-S was introduced to distinguish the stromal-derived factor from serum-derived factors with decay accelerating activity [Opferkuch *et al* (1971) originally introduced DAF as a descriptive term for the activity of the serum-derived factors]. The DAF-S designation did not endure, however, and the protein isolated by Nicholson-Weller and colleagues quickly became known simply as DAF.

In addition to its capacity to restrict the activity of the classic pathway C3 convertase, DAF was found to inhibit the activity of the alternative pathway C3 convertase (Nicholson-Weller *et al*, 1982). Thus, the functional properties of DAF suggested that its absence from erythrocytes would lead to greater C3 convertase activity and hence to greater C3b deposition on the cell surface whether complement was activated by the classic or the alternative pathway. Absence of DAF could therefore account for the observed differences between PNH and normal erythrocytes. Nicholson-Weller *et al* (1983) reported that PNH erythrocytes were deficient in DAF. Those investigators also presented data suggesting that PNH II cells were partially deficient while PNH III cells were completely DAF deficient. Almost simultaneously, Pangburn *et al* (1983) presented both functional and immunochemical evidence of DAF deficiency in PNH. The discovery of this deficiency was a

major milestone in the journey towards understanding the basis of the haemolysis of PNH that began with the observations of Strübing, Hijmans van den Berg and Ham.

Deficiency of DAF provided a logical explanation for the greater binding of C3 to PNH erythrocytes when complement was activated, and a causal role for DAF in the pathophysiology of PNH was readily accepted. But could DAF deficiency explain all the aberrant interactions of complement with PNH erythrocytes? As noted above, compelling evidence indicated that regulation of the MAC was abnormal on PNH erythrocytes (Rouault *et al*, 1978; Packman *et al*, 1979; Parker *et al*, 1985). Thus, if DAF deficiency alone were sufficient to account for the lytic sensitivity of PNH erythrocytes, DAF would have to play a role (direct or indirect) in the regulation of the MAC. Detailed studies, however, demonstrated that DAF had no inhibitory activity in the process of reactive lysis (Shin *et al*, 1986; Medof *et al*, 1987). These results implied that another protein that functioned as a regulator of the MAC must also be deficient in PNH.

Identification of the erythrocyte membrane protein that inhibit the MAC

Inasmuch as DAF deficiency could not account for the abnormal regulation of the MAC on PNH erythrocytes, attention was focused on identifying an erythrocyte membrane protein that inhibited the lytic activity of the MAC (C5b-9). Schönemark *et al* (1986) and Zalman *et al* (1986) reported the isolation from normal human erythrocytes of a protein that regulated susceptibility to reactive lysis (reactive lysis is a form of complement-mediated lysis in which the MAC is assembled on the indicator cells in the absence of membrane-associated C3 or C5 convertase activity). This inhibitory protein called C8-binding protein (C8bp) by Schönemark *et al* (1986) and homologous restriction factor (HRF) by Zalman *et al* (1986) was reported to block formation of the MAC by binding to C8, C9 or both. In 1987, each group reported that C8bp/HRF was deficient in PNH (Hänsch *et al*, 1987; Zalman *et al*, 1987). Thus, it appeared that the basis of the aberrant interaction of the MAC on PNH erythrocytes had been identified.

In 1989, however, Holguin and colleagues reported the isolation of a protein from normal human erythrocytes that inhibited reactive lysis of PNH erythrocytes (Holguin *et al*, 1989a). This protein, which they named membrane inhibitor of reactive lysis (MIRL), was clearly discrete from C8bp/HRF. Numerous subsequent studies have confirmed that MIRL deficiency accounts for the aberrant regulation of the MAC by PNH erythrocytes, and the MIRL protein and gene have been characterized in great detail (Parker, 2000). In contrast, even the existence of C8bp/HRF is in doubt because there is no data on the primary structure of the protein and neither a candidate cDNA nor a gene has been identified.

The protein designated MIRL by Holguin *et al* (1989b) was identified independently by several groups, although none of those other investigators were attempting to identify the factor that accounted for the abnormal sensitivity of

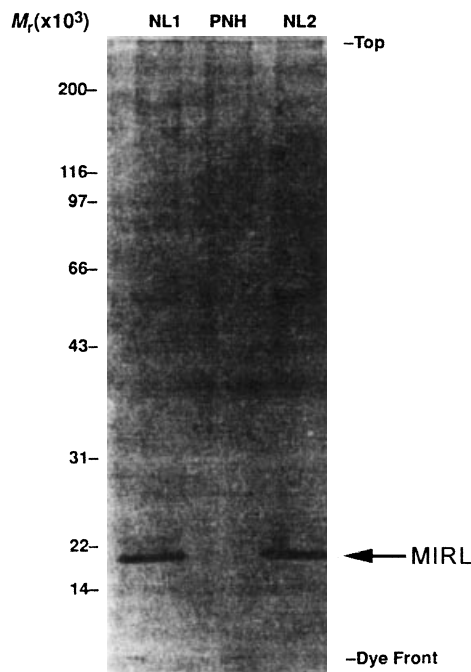


Fig 6. MRL is deficient in PNH. Analysis by Western blot of normal (NL) and PNH III erythrocyte membrane proteins using anti-MRL (CD59) as the primary antibody. A band, representing a protein with a molecular mass of ~ 18 kDa, is present in the lanes containing normal erythrocyte membrane proteins (NL1 and NL2). A band with this mass is absent in the lane containing the PNH III erythrocyte membrane proteins (PNH). Deficiency of DAF (CD55) and MRL (CD59), two GPI-anchored complement regulatory proteins, accounts for the abnormal sensitivity of PNH erythrocytes to complement-mediated lysis [reproduced from Holguin *et al* (1989a) by copyright permission of The American Society of Clinical Investigation].

PNH erythrocytes to reactive lysis. A number of different names have been proposed for the protein (e.g. CD59, protectin, MACIF, HRF 20, MEM-43, H19). By default, the protein is now referred to most commonly as CD59. For the purposes of this historical review, however, the term MRL is used because that was the name given to the protein that was absent from PNH erythrocytes (Fig 6) and which accounted for their abnormal sensitivity to MAC-induced lysis (Holguin *et al*, 1989a).

Molecular basis of the erythrocyte phenotypes of PNH

Isolation of MRL made possible methods to determine the molecular basis of the difference between PNH II and PNH III erythrocytes. Those studies showed that the two phenotypes have the same basic defect (i.e. deficiency of DAF and MRL) with the PNH III cells being more severely affected (Holguin *et al*, 1989b). On normal erythrocytes, MRL is approximately 10 times more abundant than DAF. Deficiency of expression of DAF and MRL is, in general, concordant on PNH erythrocytes. PNH II cells have approximately 10% of the amount of DAF and MRL expressed by normal erythrocytes, while PNH III cells are

completely (or almost completely) deficient. The amount of MRL found on PNH II cells is sufficient to inhibit the lytic active of the MAC in reactive lysis systems (Holguin *et al*, 1989a), but this amount of DAF is insufficient to control the activity of the C3 convertase when complement is activated by either the classic or the alternative pathway. Currently, the erythrocyte phenotypes of PNH are defined by flow cytometric analysis using anti-DAF and anti-MRL as primary antibodies. This technique is particularly informative because it clearly separates the different phenotypes based on expression of DAF and MRL and depicts the mosaicism that is characteristic of PNH (Fig 7).

Thrombophilia

Reports of thrombosis in patients with PNH appeared in the early part of the 20th century (Rosse, 2000). However, it was Crosby's exhaustive review of the clinical manifestations of the disease (Crosby, 1953a) that focused attention on thromboembolic complications as a major cause of morbidity and mortality in PNH (24/53 deaths reported in that study were a consequence of thrombosis). Hartmann and colleagues also made important contributions to the clinical description of the hypercoagulable state in PNH, in particular the relatively common association with hepatic vein thrombosis (Budd–Chiari syndrome) (Peytremann *et al*, 1972). The thrombophilia of PNH is characterized by venous thrombosis occurring at unusual sites (e.g. cerebral, dermal, hepatic, portal, mesenteric and splanchnic veins). Limited epidemiological studies suggest possible variability in the incidence of thromboembolic complications based on undefined geographical factors, ethnic factors or both (Sloand & Young, 2000).

The fundamental basis of the thrombophilia of PNH is unknown, and a consensus hypothesis describing the mechanism underlying this critically important clinical manifestation of the disease has not emerged (Sloand & Young, 2000). In his 1963 review of PNH, Dacie listed the following five problems connected with PNH: (1) The nature of the red-cell defect. (2) The nature of the factors in normal plasma which bring about haemolysis of the PNH red cell. (3) Whether the patient's leucocytes and platelets are abnormal. (4) The relationship between PNH and thrombosis. (5) The ultimate problem – the aetiology of the disease and its relationship to marrow failure. The first three problems (and a number of others) have been solved and, in the process, a remarkable amount of basic information has accrued. The last two problems (that include the relationship between PNH and thrombosis) remain challenges for the future.

PNH and marrow hypoplasia

Dacie and Lewis (1961) first drew attention to the relationship between PNH and aplastic anaemia. Numerous subsequent studies have confirmed the association. Dacie noted that 7 of his 48 cases were 'first confidently diagnosed' with aplastic anaemia (Dacie, 1963). He went on to state the following: 'I believe that these patients were in fact suffering from aplastic anaemia and that PNH developed as a complication. The odds that the two rare

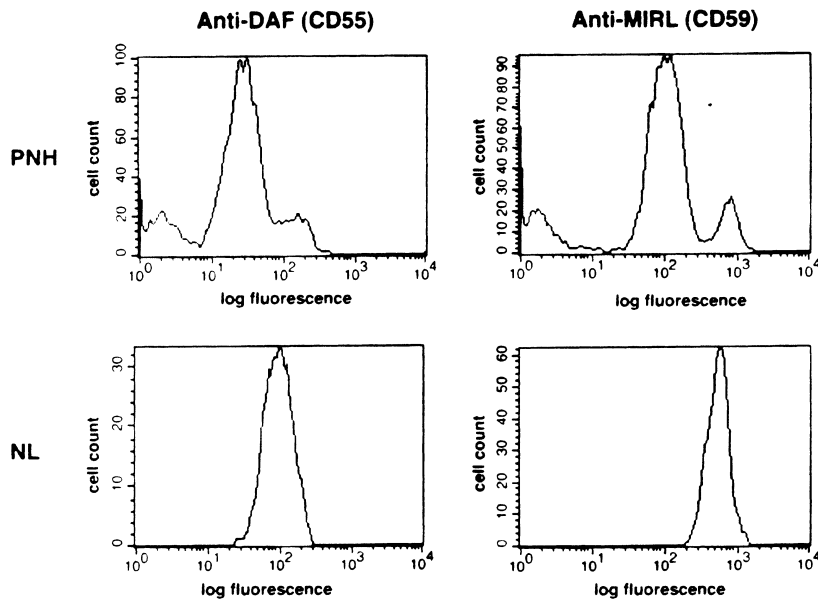


Fig 7. Demonstration of phenotypic mosaicism in PNH using flow cytometry. Erythrocytes from a patient with PNH and a normal volunteer (NL) were analysed using either anti-DAF (CD55) or anti-MIRL (CD59) as the primary antibody. The histograms from the normal donor show uniformly positive staining with both antibodies, findings consistent with a single population of cells. The histogram from the PNH patient shows three discrete populations of cells: a negative population (PNH III); a population with dim-positive expression (PNH II); a population with normal expression (PNH I) [modified from Parker (1996) by copyright permission of AlphaMed Press].

diseases could occur in the same patient as the result of chance are astronomical – they must be causally related.’ He went on to speculate that ‘It seems to me to be possible that the PNH change, resulting perhaps from somatic mutation, may be particularly likely to occur in damaged marrows, perhaps where early or abortive attempts at regenerative haemopoiesis are occurring’.

In making his case for a causal relationship between aplastic anaemia and PNH, Dacie noted in the same review: ‘Of particular significance is, I feel, the fact that the sequence of marrow aplasia progressing to PNH has been seen in at least one patient with aplastic anaemia of familial origin (a case of Fanconi anaemia reported by Dacie and Gilpin, 1944), and in probable drug-induced marrow aplasia, too, as well as in aplastic anaemia of unknown origin’. Thus, the relationship between PNH and aplastic anaemia suggested to Dacie the possibility that, in the setting of marrow hypoplasia, cells with the ‘PNH change’ resulting from somatic mutation had ‘some, as yet not understood biological advantage’. Dacie’s remarkably clear vision continues to guide research into the basis of the selective advantage and clonal dominance of the mutant haematopoietic stem cells of PNH. Conceptually, the relationship between PNH and aplastic anaemia is critically important because it provides a framework for solving the fifth problem proposed by Dacie: ‘The ultimate problem – the aetiology of the disease and its relationship to marrow failure.

Deficiency of GPI-anchored proteins in PNH

Beck and Valentine (1951) reported that neutrophils from patients with PNH are deficient in alkaline phosphatase. This observation appears to have been serendipitous as PNH leucocytes were included along with white cells from other ‘miscellaneous haematologic conditions’ as part of a study directed mainly at analysis of cells from patients

with chronic lymphocytic leukaemia and acute leukaemia. In 1959, PNH erythrocytes were found to be deficient in acetylcholinesterase (Auditore & Hartmann, 1959) and, in other studies, 5’ nucleotidase was shown to be deficient on PNH lymphocytes. The discovery that PNH cells are deficient in these three proteins was not a consequence of hypothesis-driven research but rather arose from descriptive studies. These survey experiments are often lightly regarded but, in the history of PNH, observations generated by such non-hypothesis-driven experiments proved pivotal.

Despite their archival nature, the reports of deficiency of alkaline phosphatase, 5’ nucleotidase and acetylcholinesterase continued to resonate among investigators interested in PNH. Careful studies by Kunstling and Rosse (1969) suggested a pathological link between acetylcholinesterase deficiency and PNH by demonstrating that the complement-sensitive erythrocytes lacked acetylcholinesterase activity while the complement-insensitive red cells had normal activity. That haematopoietic cells from patients with PNH were deficient in at least three membrane constituents argued that the underlying basis of the deficiency was not a consequence of mutations affecting the structural genes that encode the individual proteins. Rather, the observation was more consistent with the hypothesis that the deficient proteins share a common biochemical property that affects membrane expression and that this common feature is defective in PNH. Regardless of the exact details of the deficiency, the important point that arose from these serendipitous findings was that any hypothesis that attempted to explain PNH had to account for deficiency of multiple proteins.

In 1980, it was proposed that alkaline phosphatase was attached to the plasma membrane via a covalent linkage to a lipid molecule, phosphatidylinositol (Low & Zilversmit, 1980). This idea was not widely accepted at the time but, by

the early spring of 1985, it was becoming clear that several other cell surface proteins from a diverse set of cell types were attached to the membrane by a phosphatidylinositol anchor. Included among the first phosphatidylinositol-anchored proteins were acetylcholinesterase, 5' nucleotidase, Thy-1 antigen and the variable surface glycoprotein from trypanosomes. An interesting and particularly useful property of this anchor was that it could be released from the cell surface as a consequence of cleavage by a highly specific bacterial phospholipase, phosphatidylinositol-specific phospholipase C (PI-PLC) (Low & Finean, 1977).

A brief summary of the observations of some of the early contributors to the phosphatidylinositol anchor hypothesis was published as a news article in *Science* in 1985 (Kolata, 1985) (Fig 8). A sentence from this report reads as follows: 'But Low's enzyme cleaved phosphatidylinositol and released three unrelated membrane proteins – acetylcholinesterase, 5' nucleotidase, and alkaline phosphatase'. To an investigator familiar with the deficiency of these same three proteins in PNH, reading this summary would certainly have resulted in one of those rare eureka moments when suddenly everything makes sense.

Among those who recognized the importance of the findings were members of Dr Victor Nussensweig's laboratory at New York University Medical Center. Using enzyme provided by Dr Low, those investigators reported in 1986 that DAF is released from the cell membrane by PI-PLC (Davitz *et al.* 1986). As a consequence of those observations, the following hypothesis was proposed for PNH: all proteins that are deficient on the haematopoietic cells of

PNH are GPI-anchored; (and the corollary of this argument) all GPI-anchored proteins that are expressed by haematopoietic cells are deficient in PNH. This hypothesis was met with enthusiasm because it provided a simple yet plausible explanation for the deficiency of multiple proteins in PNH.

Deficiencies of leucocyte alkaline phosphatase, erythrocyte acetylcholinesterase and lymphocyte 5' nucleotidase account for none of the clinical manifestations of PNH. Regardless, description and awareness of these abnormalities lead directly to discovery of the basis of one of the most remarkable characteristics of the disease (GPI-anchor protein deficiency) and provided the insight needed to identify the genetic abnormality that underlies PNH.

PIG-A and PNH

By 1987, the complex structure of the glycosyl phosphatidylinositol anchor had been fully elucidated. Clearly the metabolic pathways necessary for synthesis of the anchor, post-translational modification of the proteins that are GPI-anchored, and expression of the GPI-anchored constituent on the membrane surface would require multiple enzymes and cofactors (at least nine enzymes are required for synthesis of the GPI moiety alone). Hypothetically, disruption of any component of this intricate pathway occurring in a pluripotent haematopoietic stem cell would result in the PNH phenotype. Indeed, some early biochemical studies into the metabolic basis of the deficiency of GPI-anchored proteins in PNH suggested a heterogeneous aetiology. A remarkable series of observations, however, revealed that all cases of PNH result from the mutation of a single gene.

The critical experiment (reported in 1993) that led ultimately to the genetic basis of the protein deficiency in PNH utilized a well-characterized set of cell lines that was developed at a time when the GPI-anchor was unknown. That these reagents played such a critical role in revealing the genetic basis of PNH is an example of how serendipity and insight often converge in science to yield startlingly beautiful results.

A wealth of information about the genes involved in the synthesis of the GPI anchor accrued from the careful study of Thy-1⁻ mutants derived from a murine lymphoma cell line by Robert Hyman at the Salk Institute. The mutants were generated during the early and mid-1970s to investigate by somatic gene analysis the molecular events involved in the expression of cell surface molecules (Hyman, 1985, 1988). Serendipitously, the membrane protein selected for study by Hyman was Thy-1. He isolated Thy-1⁻ mutants and rigorously characterized, using cell fusion experiments, nine discrete complementation groups (designated class A–I). In 1985, Thy-1 was shown to be GPI-anchored, and subsequent studies by several different groups identified the biochemical defect in most of the mutants. That Thy-1⁻ mutants might be informative reagents for the study of PNH was a pivotal concept that pointed straight to the genetic origin of PNH.

In 1993, Taroh Kinoshita (Fig 9) and members of his laboratory (particularly Junji Takeda) in Osaka, Japan, made a remarkable series of conceptual and technical leaps. Those investigators took advantage of mosaicism in PNH by

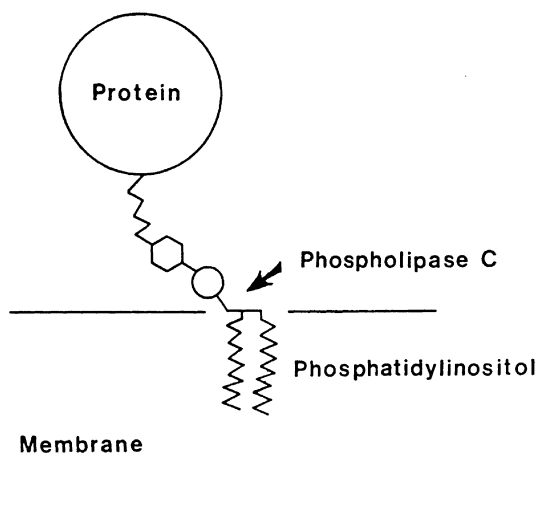


Fig 8. The phosphatidylinositol anchor. This illustration, drawn by Dr Martin Low, accompanied a news article published in *Science* (Kolata, 1985). The figure legend, authored by Dr Low, but excluded from the article, read as follows: 'A protein molecule is covalently attached to a phosphatidylinositol molecule situated in the membrane. Cleavage of the phosphatidylinositol by phospholipase C releases the protein from the membrane'. The publicity from this article led to the discovery that all the proteins that are deficient in PNH are glycosyl phosphatidylinositol anchored (the illustration was provided by Dr Low and used with his permission).



Fig 9. Taroh Kinoshita (1951–). In 1993, Dr Kinoshita and members of his laboratory at Osaka University presented incontrovertible evidence that mutant *PIG-A* accounts for the deficiency of GPI-anchored proteins in PNH. His group also demonstrated that the *PIG-A* mutations in PNH are somatic and that they occur in a pluripotent haematopoietic stem cell. They went on to show that *PIG-A* is located on the X-chromosome. These seminal observations lead to numerous studies that have defined the genetic basis of PNH (this photograph was provided by Dr Kinoshita and used with his permission).

developing paired cell lines derived from patients. Following Epstein–Barr virus (EBV) immortalization of B-lymphocytes, cells deficient in expression of GPI-anchored proteins (here designated GPI⁻) were isolated and cloned. From the same patient, cells with normal expression of GPI-anchored proteins (here designated GPI⁺) were similarly developed. Using the GPI⁻ sets from these well-characterized cell lines, Kinoshita and colleagues analysed expression of the GPI-anchored protein, CD59, following cell fusion with Thy-1⁻ mutants from complementation classes A, B, C, D, E, F and H (Takahashi *et al.*, 1993). They observed complementation of expression of CD59 by classes B, C, D, E, F and H but not by class A.

The initial reaction in the synthesis of the GPI-anchor is generation of *N*-acetylglucosamine phosphatidylinositol from phosphatidylinositol. Based on biochemical studies, others had shown that this first step was defective in cells belonging to complementation class A (this step is also defective in classes C and H mutants, but, as noted above, the somatic hybridization experiments performed by Kinoshita's group showed complementation of the PNH defect by cells from these two classes). Analysis of manno-lipid biosynthesis confirmed that GPI⁻ cells failed to

synthesize *N*-acetylglucosamine phosphatidylinositol. In contrast, synthesis of this moiety was observed when the paired GPI⁺ cells were analysed. These technically demanding studies strongly suggested that at least some cases of PNH are a consequence of mutation of the same gene that is defective in the complementation class A, Thy-1⁻ mutant originally developed by Hyman.

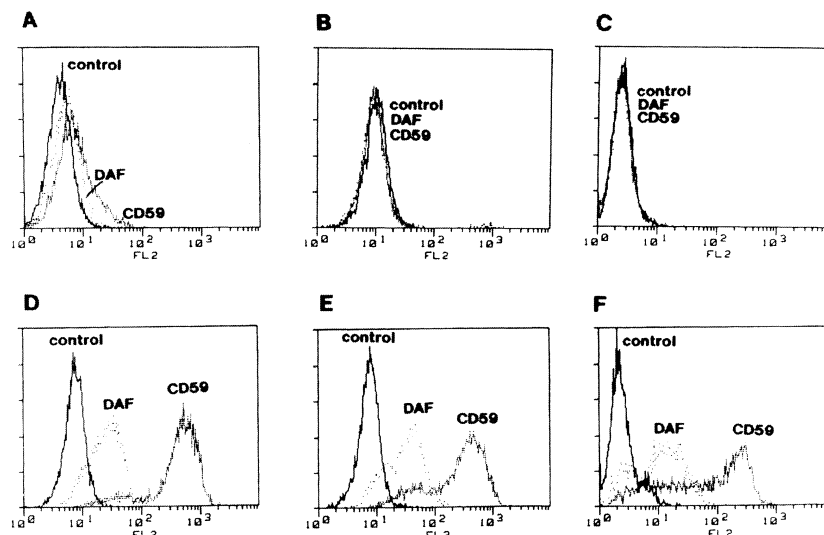
To identify the gene that is defective in complementation class A, Kinoshita and colleagues used expression cloning (Miyata *et al.*, 1993). The gene that complemented expression of MRL (CD59) on a human lymphoma cell line (JY-5) belonging to complementation class A was cloned and given the clever name, *PIG-A* (for phosphatidylinositol glycan-class A). The authors also showed that transfection with *PIG-A* restored synthesis of *N*-acetylglucosamine phosphatidylinositol in the class A mutants.

Next, the Osaka group demonstrated that deficient expression of GPI-anchored proteins was complemented when *PIG-A* cDNA was transfected into PNH cell-lines (Takeda *et al.*, 1993) (Fig 10). As anticipated, discrete mutations were observed in *PIG-A* from the GPI⁻ PNH cell lines (Takeda *et al.*, 1993). Takeda, Kinoshita and colleagues also showed that the mutations were somatic by demonstrating absence of the *PIG-A* mutation in the paired GPI⁺ cells from the same patients. That the mutation arose in a pluripotent stem cell was strongly supported by the observation that the same *PIG-A* mutation found in the B-lymphocyte cell line could be demonstrated in the peripheral blood neutrophils of the patient from whom the cell line was derived. Finally, they showed that *PIG-A* is X-linked. This finding must have been especially rewarding to the investigators because it provides a simple but compelling reason why the PNH phenotype is always a consequence of mutations of *PIG-A*. Assuming that all other genes involved in synthesis of the GPI-anchor are autosomal (this assumption is supported by available data), inactivating mutations would have to occur on both alleles to cause a deficiency of GPI-anchored proteins. However, because *PIG-A* is X-linked, only one inactivating mutation is required to produce the PNH phenotype. Further, females and males are at equal genetic risk for developing the disease because it arises as a result of a mutation in a somatic tissue (i.e. the haematopoietic stem cell) in which only one X-chromosome is active (as an interesting historical aside, Hyman (1980) reported experiments that strongly suggested that the gene that is mutant in complementation class A is X-linked).

The discovery by Kinoshita and colleagues that PNH is a consequence of mutant *PIG-A* was a watershed event. To date, a genetic basis for PNH other than mutations in *PIG-A* has not been reported [the single case of isolated, inherited deficiency of MRL in a patient with the PNH phenotype (Yamashina *et al.*, 1990) is excluded because it does not fit the PNH paradigm]. On the other hand, more than 150 unique mutations in *PIG-A* have been identified (Luzzatto & Nafa, 2000).

Characterization of the genetic basis of PNH also made possible studies that defined the aetiology of the phenotypic mosaicism that is one of the most remarkable characteristics

Fig 10. Complementation of deficient surface expression of DAF and MIRL (CD59) on PNH cells. Three GPI anchor-deficient cell lines derived from B-lymphocytes of patients with PNH (panels A, B and C) were stained with anti-DAF and anti-MIRL (CD59) and analysed by flow cytometry. The same cell lines transfected with *PIG-A* cDNA (panels D, E and F) were analysed under the same conditions as the controls. These experiments implied that *PIG-A* is the gene that is mutant in PNH. That mutations in *PIG-A* account for deficiency of GPI-anchored proteins in PNH was demonstrated by Dr Kinoshita and colleagues in this same paper [reprinted from Takeda *et al* (1993) with permission from Elsevier Science].



of PNH. Endo *et al* (1996) cloned, from a single patient, T lymphocytes that varied in expression of GPI-anchored proteins. They found that each of the four clones analysed harboured a discrete *PIG-A* mutation. Mutations predicted to abolish *PIG-A* function were found in cells with absent GPI-anchor protein expression, while cells with partial expression were found to have a missense mutation hypothesized to reduce, but not abolish, *PIG-A* function (Table III). Thus, the phenotypic mosaicism of PNH is a consequence of genotypic mosaicism.

The future

The genetic basis of PNH has been identified unequivocally (Takeda *et al*, 1993). Mutation of *PIG-A* accounts for deficiency of GPI-anchor protein expression in PNH, which in turn clearly explains the abnormal sensitivity of the affected erythrocytes to complement-mediated lysis. However, the *PIG-A* mutation does not explain why the mutant stem cell clones expand and, in many cases, dominate haematopoiesis. Understanding the genetic basis of haematopoietic malignancies such as chronic myelogenous leukaemia (CML) and various subtypes of acute leukaemia and non-Hodgkin's lymphomas usually provides a plausible explanation for the

clonal dominance of the mutant cells. A good example is expression of the BCR-ABL fusion protein in CML that is a consequence of t(9;21). Such is not the case with PNH, however, because deficiency of GPI-anchored proteins endows the cells with no obvious growth or survival advantage. That multiple *PIG-A* mutant clones can be identified in a single patient, however, suggests a powerful selection process is at work on the bone marrow. It is the nature of that selection process that is the target of ongoing research. Whatever the aetiology of the selection pressure, it must involve deficiency of one or more GPI-anchored proteins.

Thus, current investigators are focusing their efforts on the fifth problem identified by Dacie in his 1963 review: 'The ultimate problem – the aetiology of the disease and its relationship to marrow failure'. When this history is updated in a decade, chronicling of experiments leading to the solution of Dacie's ultimate problem will almost certainly be included (I'm less certain about inclusion of a satisfying explanation for the thrombophilia of PNH, Dacie's fourth problem).

What will be the answer to the aetiology of the clonal dominance? Might it be an GPI-anchored protein expressed by haematopoietic stem cells that is the target of cytotoxic

Table III. PNH phenotypes.*

Phenotypic designation	Complement sensitivity†	GPI-anchor protein expression by flow cytometry‡	Type of <i>PIG-A</i> mutation (effect on protein)
PNH I	Normal	Normal	None
PNH II	Moderate sensitivity (3–4 times more sensitive than normal)	Dim Positive	Missense (partial inactivation of <i>PIG-A</i>)
PNH III	Markedly sensitive (15–20 times more sensitive than normal)	Negative	Nonsense, frameshifts, deletions, insertions (complete inactivation of <i>PIG-A</i>)

*Modified from Parker & Lee (1998).

†Based on the complement lysis sensitivity assay of Rosse and Dacie (1966) (illustrated in Figs 4 and 5).

‡Based on flow cytometry of erythrocytes (illustrated in Fig 7).

T cells? Will a negative regulator of haematopoiesis that is GPI-anchored be identified? Are mutations affecting genes other than *PIG-A* necessary for clonal expansion? Perhaps we will come full-circle and find that regulation of complement modulates haematopoiesis or that the haemolytic anaemia itself is the driving force behind clonal selection? Will the solution to the ultimate problem finally provide an explanation for the nocturnal component of the haemoglobinuria? The most intriguing possibility, however, is that the basis of the clonal dominance of *PIG-A* mutant stem cells is something as yet incompletely imagined. That has happened before with PNH.

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