Flattery (or unprincipled Complement) PIGs without tails, and Lost Spelunkers:

The Story of Paroxysmal Nocturnal Hemoglobinuria

UT-Southwestern
Medical Grand Rounds
November 16, 1995

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I. Presentation of a case

The patient is a 49 y.o. black woman who first became ill after one of her six spontaneous abortions in the 1969. By second hand descriptions, she presented elsewhere with fever, chills, and dark urine. The latter persisted for some "weeks" after the fever and chills subsided. Two to three times per year, she had attacks of left upper quadrant pain lasting several days. In 1971, she presented with diffuse abdominal pain, an obviously enlarged spleen, fever, chills, and dark urine. Her hematologist believed her have β-thalassemia and spherocytosis. The details of her evaluation at that time are not available. Later in 1971, she was evaluated for severe headache, had an elevated opening pressure on lumbar puncture, and was felt to pseudotumor cerebri. In 1977 she presented to PMH apparently with abnormal LFTs. Liver biopsy showed passive congestion only. No records were available from that admission. In 1980, she represented to PMH with ascites, "abnormal LFTs", and venography proved hepatic vein thrombosis confirming the diagnosis of Budd-Chiari syndrome. She was admitted to Dr. Foster's inpatient service and a persistent MSIII (Al Smith) obtained the first history of intermittent red or dark urine. This was the critical clue to the diagnosis of paroxysmal nocturnal hemoglobinuria. During that admission, she was noted to be pancytopenic, her Ham test was positive, and her urine was positive for hemosiderin. (see figure 1 for lab data) The leukocyte alkaline phosphatase was 8 with normal being > 150. The latter three findings established the diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). Most notes suggest that her family history (10 siblings) was negative but Dr. Ware confirms (as does she) that her brother developed PNH and ultimately died of thrombotic pancreatitis. She was diagnosed to have a bicornate uterus and that was considered to be the cause of her $G_6P_0SAb_6$ reproductive history. Her ascites proved extremely difficult to manage prompting a side-to-side porta-caval shunt. This procedure failed due to clotting in the portal vein and she then underwent successful placement of a Denver shunt. The Denver shunt drained ascites into the circulation without complication for several years but no longer functions. She was admitted in the 1980s on multiple occasions for symptomatic anemia requiring transfusion, infection-induced hemolysis, and once for deep venous thrombosis of the right leg. Treatment has focused on hematinic agents including multivitamins, folate, and iron. Every other day prednisolone therapy was begun in 1985 and decreased her hemolysis. She has also been on Prednisolone 10-20 mg qod for almost 15 years. In 1986, she was found to practice geophagia in kilogram amounts per month. Her pancytopenia, creatinine, and ascites are currently stable though she complains recently of increasing nosebleeds.

Test	Apr-80	Apr-81	Aug-82	Apr-83	Oct-86	Jul-87	Aug-88	Mar-91	Feb-93
Hgb		8.8	8.8	7.6	5.6	9.6	11.8	11.3	13.2
Hct		27	27	24	16.7	30	37	35	39.8
MCV		96	92	92	98.7	92	93	86	83
WBC		1.2	1.8	1.7	1.3	2.3	3.4	2.7	4.4
Plts		51	68	67	22	52	80	59	70
MPV					8.8	14.4	12.1		
Creat		0.5		0.6	0.7	0.7	0.6	0.6	0.8
Alb		4.6	4.2	4.6	4.2	4.1	4.4	4.1	4.2
Bili	1.8	1.6	1.3	1.3	1.4	1.4	1.5	0.9	1
AST	60	83		58	63	38	55	49	43
AlkP		142		126		179	207		203
LDH									
Fe									
TIBC									
LAP	8								
	NI								
Retic					0.049				

Figure 1- Laboratory data for S.M.A

II. History of PNH

In 1866, Gull described the first case of PNH in Guy's Hospital Reports(1). (see figure 2) The case report did not lead to widespread appreciation of the syndrome. Therefore, Strubing redescribed the case independently 20 years later(2). He recognized that paroxysms of hemoglobinuria were more likely to occur at night than during the day. He associated paroxysms with beer drinking but all subsequent investigators have failed to reproduce his initial speculations in this regard. He correctly believed that erythrocytes are abnormal in this condition but incorrectly believed that the abnormality caused them to pass through the kidneys into the urine.

History of PNH

1866 Gull-Described what the first case of probable paroxysmal nocturnal hemoglobinuria(PNH) but the description was inadequate to allow others to recognize the syndrome.

1882 Strubing-Offered the first clear description of PNH as a syndrome. He correctly associated the disease with nocturnal paroxysms of hemoglobinuria but incorrectly associated paroxysms with beer drinking. He also incorrectly concluded that the hemoglobinuria was caused by defective red cells passing through the kidneys into the urine.

1911 van den Bergh-Demonstrated that erythrocytes from a patient with paroxysmal icterus were easily lysed by CO2 treated serum. This was the first proof that PNH erythrocytes are abnormal.

1937 Ham-Discovered that complement was required for acid and antibody-induced lysis of PNH erythrocytes. Acid-induced lysis remains a standard means of diagnosing PNH - Ham's test.

1954 Pillemer-Discovered the alternative pathway for complement activation. This resolved one of the major controversies regarding PNH. Until Pillemer, antibody was considered essential to complement activation yet PNH erythrocytes were

Figure 2-History of PNH part I

Van den Bergh discovered in 1911 that PNH erythrocytes could be readily lysed if incubated with serum under carbon dioxide(3). This experiment proved unequivocally that PNH erythrocytes were abnormal though no clear insight was gained into what the abnormality was. Complement was only just being discovered during van den Bergh's career so it was many years before the association between lysis of PNH erythrocytes and complement was made by Ham(3-5).

Ham discovered that CO_2 induced lysis of PNH erythrocytes was due to acidification and required the action of complement. The Ham test is still the standard test used to diagnose PNH clinically. He showed that specific antibodies would also initiate lysis but his most surprising result was that antibodies were not required. At that time, all known complement activation responses required antibody and were mediated by what we now call the classical pathway. After the debate began to rage about the possibility complement activation without antibody, acid-induced and complement-mediated lysis of PNH erythrocytes remained one of the strongest arguments for the existence of an alternative pathway. Pillemer's discovery of the alternative pathway(6, 7), provided the theoretical means for explaining PNH observations and enabled Hinz to resolve the controversy(8).

Oni (see figure 3)discovered in 1970 that PNH is a clonal disease(9) paving the way for classification of erythrocytes in PNH patients by three types. I-normal erythrocytes; III-exquisitely sensitive to complement lysis; II-intermediate sensitivity to complement lysis (10, 11)(see figure 4).

Rosse and colleagues showed that PNH erythrocytes bound much greater amounts of C3 than normal cells whether the classical or alternative pathways were stimulated(12, 13). This led to the observation that PNH erythrocytes were deficient in a membrane constituent that down regulates the activity of C3 convertase. Two groups(14, 15) showed that PNH erythrocytes were deficient in

decay accelerating factor (DAF-CD55), a cell surface protein known to inhibit C3 convertase(16). In a series of critical experiments, Medof et al showed that anti-DAF antibodies caused normal erythrocytes to become sensitive to complement in the Ham test(17). Furthermore, they showed that a adding DAF-rich extract to PNH erythrocytes made them resistant to complement-mediated lysis. The extract probably was contaminated with other GPI-anchored proteins. It was tempting to conclude that the cellular defect in PNH was DAF deficiency but it was also known that PNH erythrocytes were deficient in acetylcholinesterase (AchE)(18). The biochemical link between AChE and DAF deficiency in PNH was complete when it was demonstrated that both proteins are anchored to the cell membrane by glycosyl-phosphatidylinositol (GPI) rather than a traditional transmembrane anchor(19, 20). All PNH cells are deficient in GPI-anchored proteins and all GPI-linked proteins are deficient in PNH cells.

History of PNH

1960 Auditore-Showed that PNH erythrocytes are deficient in plasma membrane acetylcholinesterase activity

1965 Lewis-Showed that a population of leukocytes in PNH patients express no alkaline phosphatase

1970 Oni proved that PNH is a clonal disorder of hematopoietic stem cells.

1974 Rosse categorizes PNH cells in three groups: I-normal erythrocytes; III-exquisitely sensitive to complement lysis; II-intermediate sensitivity to complement lysis

1977 Low-Characterized the glycosyl-phosphatidylinositol (GPI) system for anchoring proteins and showed that alkaline phosphatase has a GPI anchor

1983 Nicholson-Weller-Demonstrated that PNH erythrocytes lack decay accelerating factor (CD55) a complement regulatory protein

1989 Holguin established the preeminent role of MIRL in complement-mediated lysis of PNH erythrocytes

1993 Takeda-Identified the PIG-A gene, demonstrated it was mutated in PNH patients and localized the structural gene (PIG-A) to the X-chromosome. This provided the biochemical basis for deficiency of GPI-linked proteins in PNH.

Figure 3-History of PNH part II

PNH Cell Types

PNH I	**Normal Cells**
PNH II	**Intermediate Sensitivity**
PNH III	**Exquisitive Sensitivity**

Figure 4 PNH cells types defined by degree of complement sensitivity (10)

In 1989, Holguin et al purified the membrane inhibitor of reactive lysis (MIRL) and showed that adding it to PNH erythrocytes also prevents complement mediated reactive lysis(20). They also raised antibodies to MIRL and showed that incubation of normal erythrocytes with this antibody rendered them sensitive to complement mediated reactive lysis. These and other experiments described in section V. established the preeminent role of MIRL in the pathogenesis of PNH.

Takeda et al verified the proposed deficiency in GPI-anchor biosynthesis in PNH when they discovered that PNH patients have a somatic mutation of the PIG-A gene(21). The gene product is part of an enzyme complex, N-acetylglucosaminyl phosphatidylinositol synthase(Glc-NAc-PI), that catalyzes the first reaction in GPI-anchor biosynthesis. These investigators also showed that the defect behaves as if dominant without being inherited because PIG-A resides on the X-chromosome. Mutation of a single allele eliminates Glc-NAc-PI

synthesis activity because males have only one X-chromosome and females inactivate one X-chromosome in each somatic cell by a process called lyonization.

Initial Diagnoses and Time to PNH diagnosis

Initial Dx and # of cases (n=26)		Time to PNH DX (mos)
Hemolytic Anemia	8	0-96
PNH	7	0-6
Aplastic Anemia	4	9-84
Iron Deficiency	3	1-12
Autoimmune Hemolytic Anema	2	0
Lymphoprolerative Disorder	1	8
Crohn Disease	1	48

Figure 5-Initial diagnoses in PNH and time required to make the correct diagnosis (28).

III. Natural History of PNH

PNH can be a very difficult disease to diagnose because most patients fail to present with classical signs and symptoms. Our patient was symptomatic for many years before any diagnosis was made and even longer before the correct diagnosis was made. This is a common phenomenon as shown in figure 5.

PNH is a chronic hemolytic anemia punctuated by episodes of overt hemoglobinuria that are more likely to be noticed after sleep. The most common causes of death are related to either thrombosis or bleeding(22)(See figure 6). The venous thromboses are often in unusual locations and there is probably an increase in arterial thromboses too. PNH reduces the production of all three hematopoietic lineages to variable degrees. Thus, normal erythrocytes are produced in reduced numbers and the abnormal erythrocytes are produced in great numbers but have a markedly reduced life span. PNH is a an acquired clonal, and yet non-malignant disease of a single (usually) pluripotent/totipotent stem cell that curiously dominates the circulating erythrocyte pool in spite of a pronounced survival disadvantage in the circulation. Acute non-lymphocytic leukemia eventuates in a fraction of affected individuals (1-10%) but usually not for several years after the diagnosis of PNH is made. The protean manifestations of this interesting disease have taught us a great deal about the biology of RBC clearance, membrane anchoring schemes for proteins, the biology of aplastic anemia, and the regulation of complement.

Causes of death in 60 PNH Patients

PNH Related		**28**
Venous Thromboses		**14**
Hepatic vein	7	
Inferior vena cava	1	
Cerebral vein	1	
Mesenteric vein	2	
Pulmonary embolism	3	
Bleeding		**11**
Gastrointestinal	6	
Subarachnoid	3	
Intracerbral	2	
Miscellaneous		**3**
Liver failure	2	
Intraabdominal event	1	
PNH Unrelated		**20**
Unknown		**12**

Figure 6-The causes of death in a series of 60 are shown above. 58% of patients who died with known cause died of complications attributed to PNH (27)

A. Epidemiology

More than 280 cases of PNH have been seen at Duke alone(23). The best estimates are that the PNH frequency is between one and ten per million population in the United States(24-26). Thus, in the absence of

referrals, we could expect between 3 and 30 cases in the Dallas/Ft. Worth area.

One of the most interesting features of PNH epidemiology is that it tends to parallel the epidemiology of aplastic anemia(24, 27). This is circumstantial evidence in favor of the hypothesis that aplastic anemia is permissive for PNH.

B. Chronic Hemolytic Anemia and Hemoglobinuria

Chronic anemia and episodic hemoglobinuria accounts for about 50% of the presenting signs for patients with PNH(28). Patients become anemic through the synergistic effects of diminished production of normal RBCs (PNH I cells) and increased destruction of RBCs affected by PNH (PNH III cells--PNH II cells are partially affected). One or occasionally more clones of diseased pluripotent stem cells produces between 20% and 60% of the circulating RBCs. The remaining RBCs are derived from the normal stem cells. The normal number of stem cells (10^6 to 10^7) is such that a putative PNH clone that produces the same number of RBCs as other stem cells should account for no more than one millionth of circulating RBCs. Less than 1% severely affected PNH III cells produces no clinical disease and cannot be detected by the Ham test. Even patients with less than 20% PNH III RBCs rarely if ever have overt hemoglobinuria. Since PNH III cells have an eight day life span, their over representation in the circulation represents massive over representation in the marrow yet without malignancy (see figure 7). RBC production dynamics discussed in section V.B.3.

PNH and Normal Reticulocyte Kinetics

Consider a patient with 40% PNH III cells and a RBC count of 2.5 million

	PNH I	PNH III
RBC Life Span	100 days	8 days
% replaced/day	1%	12.5%
cells produced/day	15,000	125,000
Ratio		8.3
stem cell pool size	3,000,000	1

Figure 7-The normal stem cell pool is between 1 and 10 million. This hypothetical case has 40% of her RBCs derived from the PNH clone. All of these cells came from a single stem cell whereas the remaining 60% of RBCs are derived from a mixed population of 3 million stem cells.

Hemolysis of cells derived from the abnormal clone is due to enhanced sensitivity to autologous complement activation. This process is constant in PNH patients whether they have hemoglobinuria or not. The dramatic increase in RBC production by the diseased clone produces folate deficiency if untreated just as in other hemolytic diseases. Intravascular lysis of RBCs (unusual for intracorpuscular hemolytic diseases) decreases serum haptoglobin and increases hemoglobin in the glomerular filtrate. Renewal of the proximal tubule epithelium can result in shedding 20 mg of iron per day in PNH. This amount is 10 times the normal loss iron from the entire body and often results in severe iron deficiency. Iron deficiency can occur even in the complete absence of overt hemoglobinuria(29). The combination of iron and folate deficiencies can hide behind a normal MCV because of the mixed RBC populations produced. Patients generally require iron and folate replacement therapy to keep up with ongoing losses of these critical hematinic agents.

The disease name carries the implication that the hemoglobinuria occurs intermittently and primarily at night. The nocturnal increase in hemolysis is believed to be due bacterial endotoxin that increases in the blood at night as part of

a circadian rhythm(30). Hemoglobinuria is actually constant but it is not usually clinically apparent. Paroxysms of obvious hemoglobinuria tend to occur when intercurrent events conspire to activate complement and are more likely to afflict patients with more than 50% of their RBCs affected by the disease. Infections with either viruses or bacteria are the most common triggers for hemolytic episodes. Incompatible transfusion is a well described inciting event that is largely preventable now. As little as 60 ml of incompatible plasma contaminating platelet transfusions have been known to precipitate severe hemolysis and even renal failure(31).

C. Thrombosis

The most common cause of PNH-related death thrombosis. Thromboses typically occur in unusual veins leading serious damage of the organ(s) involved. Thrombotic occlusion of the hepatic veins(32-34) (Budd-Chiari Syndrome) or abdominal/splanchnic veins(35, 36) get these patients referred to a gastroenterologist or hepatologist. Many of these patients (like ours) give histories of chronic intermittent abdominal pain that are likely to be due to relatively minor thrombotic events. Thrombotic events may precede clinically apparent hemolysis as in the case presentation followed here in liver clinic. Thrombotic occlusion may afflict many sites including hepatic veins(22, 37), cerebral veins(38, 39) or arteries(22, 40), splanchnic veins(41) coronary arteries(22), and even skin veins(42) resembling thrombotic thrombocytopenic purpura (see figure 8). The mechanism of thrombosis is not clearly defined yet but will no doubt tie in to the fascinating biology of all the other manifestations of PNH.

Thromboses by Site in 80 PNH patients

Intraabdominal		**18**
Hepatic vein	8	
Inferior vena cava	3	
Mesenteric vein	4	
Splenic vein	1	
Renal vein	1	
Unspecified	1	
Other Venous sites		**23**
Cerebral vein	4	
Pulmonary embolism	9	
Deep vein	7	
Superficial	3	
Arterial		**8**
Myocardial infarction	6	
Cerebrovascular	2	

Figure 8-Unusual thromboses are routine in PNH patients. This figure tabulates thromboses by site in 80 PNH patients (27).

The platelets in PNH patients are subdivided into affected and unaffected elements just as with RBCs. PNH platelets have no GPI-anchored proteins(43, 44) and are clearly abnormal functionally. Complement activation has not been shown to play a decisive role in the abnormal functioning of PNH platelets that leads to thromboses. PNH platelets are more sensitive than normals to thrombin-induced aggregation by a factor of 10 to 100(30). They are not however more likely to aggregate when exposed to more typical agonists such as ADP and thromboxane A_2(30). The cause of increased sensitivity to thrombin in PNH platelets is not known but the major protein known to be abnormal in RBCs (MIRL-CD59) may play a role. This and other possibilities are discussed under section IV.

D. Aplastic Anemia

A large literature exists on the association between aplastic anemia and PNH. This disease association is the most common one with PNH(45). Patients are often diagnosed with aplastic anemia who later develop PNH but multiple

cases have been described in which the PNH precedes the diagnosis of aplastic anemia. When PNH develops in patients already suffering from aplastic anemia, the anemia and prognosis improve because the high productivity of the PNH clone improves the cell counts (see figure 9).

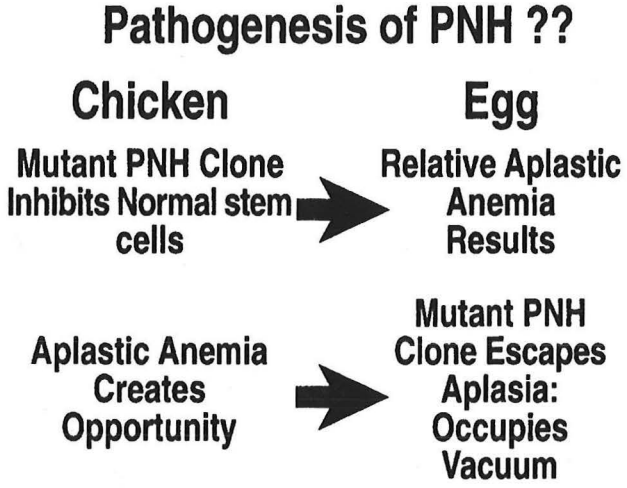

Figure 9-PNH is clearly associated pancytopenia and often overt aplastic anemia. Currently, investigators debate whether the PNH clone causes marrow aplasia (top) or aplasia allows/selects a PNH clone of stem cells.

E. Infection

Patients with PNH who get even minor infections are far more likely to seek medical attention than others. Infections activate complement and promote hemolytic complications in PNH patients. There is circumstantial evidence that PNH patients may be at increased risk for infections with adequate granulocyte counts. In a recent review of 80 patients over 50 years, not a single patient died due to a PNH related infection(22).

However, the proliferation of all normal hematopoietic cells is diminished in PNH. Granulocytes are no exception and overt granulocytopenia may result. Obviously, granulocytopenia can lead to life threatening bacterial infections. Most PNH patients have low white blood counts but not low enough to cause an increase in systemic infections. A small fraction of PNH patients, however, do have an increased frequency of bacterial infections not due to granulocytopenia(30). The current ideas about the mechanisms involved are discussed under section IV.

F. Acute Leukemia

Patients with PNH probably die from acute non-lymphocytic leukemia more often than the general population. Estimates range from as low as 1%(30) to 10% in the United States(25). In China, as many as 25% of PNH patients will die of ANLL(46). ANLL usually occurs 4-7 years after the diagnosis of PNH and is highly resistant to therapy. A recent study from England reported 80 patients followed since 1940 and the cause of death in 60. There were no deaths from acute leukemia(22).

G. Renal Failure

Increased filtration of hemoglobin and reabsorption by the proximal tubule may cause Fanconi's as the hemoglobin competes for the absorptive apparatus and ultimately damages the epithelium(47).

Renal efforts at hemoglobin reabsorption consistently cause hemosiderosis of the proximal tubule epithelium. Sometimes this condition is so severe that PNH kidneys will set off metal detectors in airports! Severe cases can cause renal failure late in the course of the disease and there are reports of acute renal failure during episodes of brisk hemolysis in PNH(48).

H. Pregnancy

Pregnancy is associated with a high mortality rate for both mother (5.8%) and fetus (30%)(49). There are reports of individual mothers successfully delivering healthy infants from multiple pregnancies(50). Generally, pregnancy is a high risk venture in PNH and should be undertaken advisedly.

I. Spontaneous remission

One of the most curious features of PNH is a tendency to spontaneous remission. In a landmark report of multi-decade follow up of 80 patients, published last week, Hillmen et al reported that 15% of their patients recovered spontaneously without explanation or subsequent relapse(22). Most of the rest of the patients however died as a result of the disease making decisions regarding high morbidity/mortality therapies like bone marrow transplantation complex at best.

J. Diagnosis of PNH

The details of the differential diagnostic evaluation of all hemolytic anemias is offered under section V. A high index of suspicion is required to make the diagnosis of PNH. Patients often present with symptoms that do not clearly indicate disease of the hematopoietic system. Typically, pancytopenia is the key initial laboratory clue to the diagnosis. Bone marrow examination usually reveals a cellular marrow that is not diagnostic. Urine hemosiderin may be positive, the leukocyte alkaline phosphatase test may be abnormal as it was in our patient, and the sugar water test may be suggestive(51) but the modern diagnosis depends on the Ham test(52). When PNH is suspected, a Ham test should be performed. The only disease known to produce a false positive Ham test is hereditary erythroblastic multinuclearity with an acidified serum lysis test (HEMPAS)(52). HEMPAS is a disease of children with a very unusual appearance of the marrow. For practical purposes, an unequivocal positive result confirms the diagnosis of PNH. The biological basis of the Ham test is discussed in section V.

IV. Biology of PNH

Increased understanding of the biology of PNH has progressively refined the diagnosis of PNH and no doubt holds the key the future therapeutic developments.

A. Complement--DAF (CD55), C8-binding protein, MIRL(CD59)

1. Introduction:

The biology of PNH has been tied to complement since Ham's first paper on the subject was published in 1937(53). This was true even when no one believed in an alternative pathway for complement activation. Complement-mediated destruction of erythrocytes remains at the heart of the clinical diagnosis of PNH so a brief review of complement activation is in order.

Complement: The complement system has many features in common in with the clotting cascade. It is composed of a series of sequentially acting proenzymes whose action can be initiated by two different schemes but ending in a final pathway common to both (see figure 10). For more information see Boackle's excellent review(54). Most of the proteins involved in the complement cascade are synthesized by the liver but some are made locally. The hydrolysis required to

start the cascade of complement reactions, can be initiated by specific antibodies (Classical) or non-specific reactions that usually depend microbial membranes or walls and on proteases derived either from microbes or inflammatory cells (Alternative pathway-see figure 11).

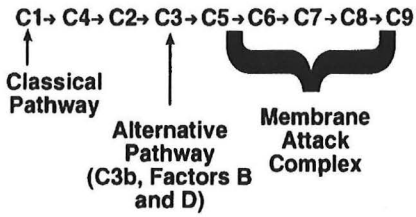

Figure 10-Complement Activation Sequence

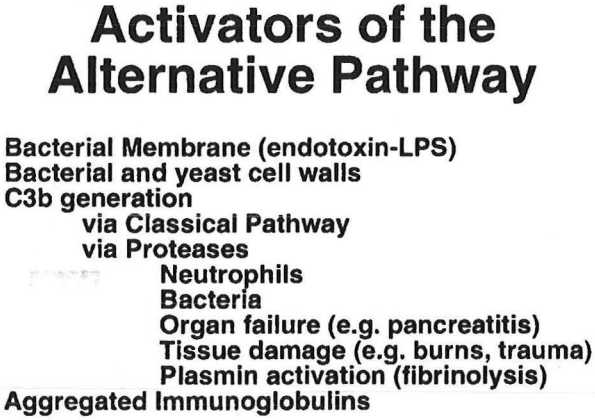

Figure 11-Summary of activators of the Alternative pathway for complement activation (54).

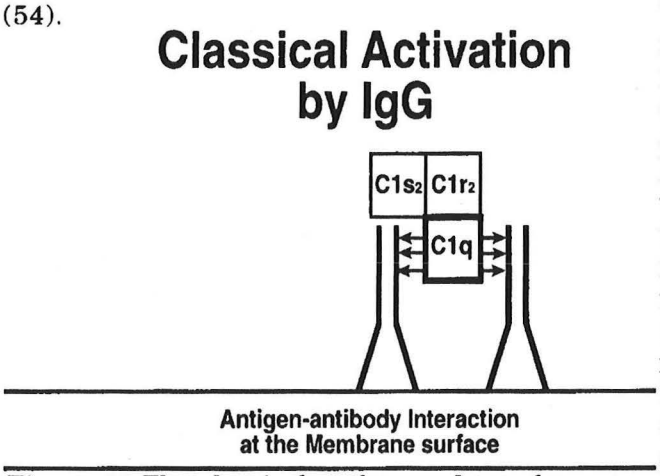

Figure 12-The classical pathway of complement activation requires juxtaposition of Fc immunoglobulin domains attached to a surface. Attachment to a surface alters Fc conformation and allowing C1q to bind and sequentially activate C1r and C1s [adapted from (54)].

2. Classical pathway of complement activation

Antibodies bound to specific antigens acquire an ability to activate complement that unoccupied antibodies do not possess. Antigen/antibody complexing induces a conformation change in the Fc portion of the antibody that exposes a C1q-binding domain. This is the initial step in the classical complement cascade. This reaction requires two Fc domains in close proximity interacting with C1q so either the antibody must be IgM or IgG on the membrane surface in sufficient density to ensure close proximity of two Fc domains. The various IgG subclasses have variable ability to participate in these reactions. IgG_3 > IgG_1 > IgG_2 and IgG_4 has not activity at all. Two C1r and two C1s molecules bind to C1q after its' association with the antibody domains. (see figure 12) This reaction depends on Ca^{2+}. Activated C1s cleaves C4 to release C4a and C4b. The "b" in complement notation refers to molecules capable of binding to membranes or other complement components while the "a" denotes soluble molecules that are released. Newly formed C4b has a binding site with a short half-life (nanoseconds) that enables it to attach only to nearby membranes. C4b production is the first major amplification step because activated C1s produces many C4b molecules. Activated C1s also cleaves C2 into C2a and C2b. C2a is released and C2b binds to C4b in a Mg^{2+} dependent reaction (see figure 13). Thus, EDTA blocks complement activation by the classical pathway by preventing C2b and C4b from binding and by preventing the association of C1q with C1r and C1s. The active protease,C4b2b, is also called C3

convertase activity.

The well known C1 inhibitor inactivates loosely associated soluble complexes of activated $C1r_2$ and $C1s_2$. Deficiency of C1 inhibitor causes angioneurotic edema.

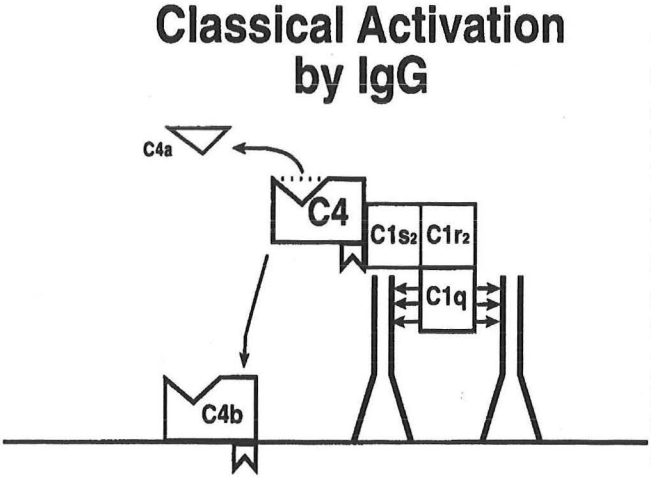

Figure 13-C4 activation-Activated $C1s_2$ cleaves C4 releasing C4a and briefly exposing a membrane binding domain on C4b. The short C4b half-life decrease damage to bystander cells [adapted from (54)].

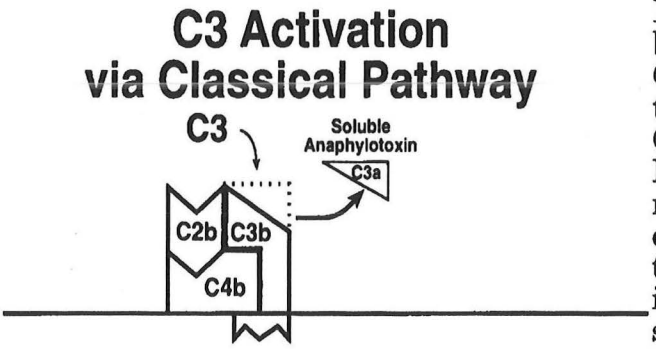

Figure 14-C3 activation-Bound C4b cleaves C2 and binds to C2b. The activated C4b2b complex cleaves C3 and C3b attaches to the complex and the membrane. This step amplifies the system [adapted from (54)].

Activated C4b2b molecules hydrolyze thousands of C3 molecules to C3a (soluble) and C3b (bound). C3b attaches to either the nearest membrane or to the C4b2b (see figure-14) complex while C3a is released as an anaphylotoxin. C3b binding to any large structure (bacterium, cell membrane fragment etc) enables neutrophils to phagocytose the structure. Enhancement of phagocytosis by this and related mechanisms is called opsonization. Cells defend themselves against C3b deposition by expressing decay accelerating factor (DAF-CD55). DAF inhibits C3b binding to host cells.

3. <u>The Membrane attack complex (MAC)</u>

C5 is hydrolyzed by the activated complex $C4b2b3b_n$ as shown. Initially, both C5a and C5b are soluble but C5b forms a soluble complex with C6 and C7 that ultimately attaches to the cell membrane (see figure 15). The C5b-6-7 complex is a receptor for C8. Remarkably, upon association with membrane bound C5b-6-7, the α and β domains of C8 undergo transmembrane insertion. As shown in the figure, this complex provides the scaffold for spontaneous polymerization of C9 molecules into a pore. The pore has a 100 angstrom diameter (10 nm) and constitutes the membrane attack complex (MAC). The MAC creates therefore a large water filled channel in the cell membrane eliminating the ability of the membrane to exclude solutes. Sodium ions, calcium ions, and other solutes diffuse rapidly into the cell down concentration gradients and increase the osmolality of the intracellular fluid. This allows water to enter the cell using the entire membrane surface inducing rapid swelling and lysis.

There are a series of proteins that prevent complement from lysing normal human cells. These include CR1 receptor glycoprotein which binds to C3b inhibiting its' activity and sensitizing it to factor I inhibition. Decay accelerating

factor (CD55), homologous restriction factor (C8-binding protein), and membrane inhibitor of reactive lysis (MIRL-CD59) also modulate complement activity on host cells to prevent self-lysis. DAF and MIRL play key roles in PNH especially MIRL.

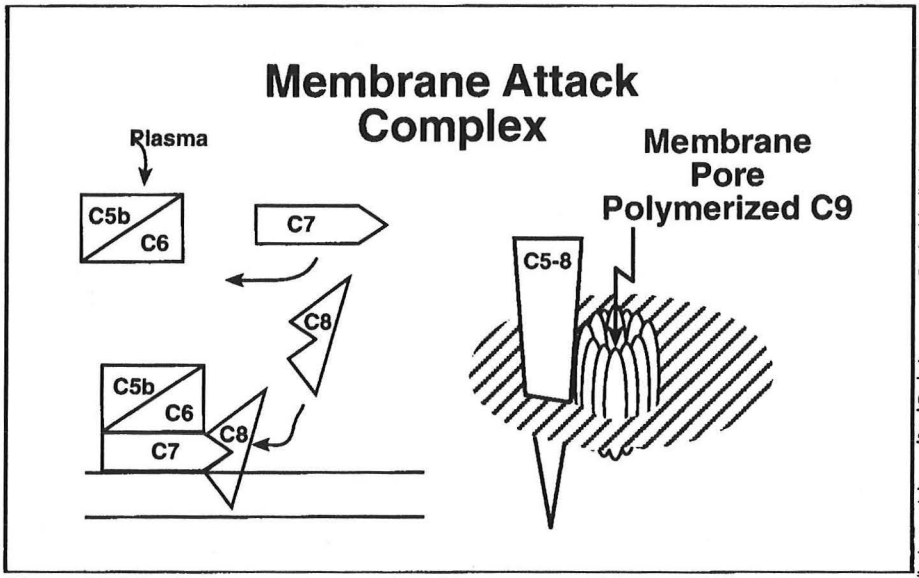

Figure 15-Membrane Attack Complex-C3 convertase cleaves C5 into C5a and C5b. C5b sequentially binds C6 and C7 in plasma. When this complex binds C8, part of the C8 protein inserts into the membrane. C5b678 polymerizes C9 insertion into the membrane forming a pore as shown. Solutes rushing into the cell cause osmotic lysis [adapted from (54)].

4. Alternative pathway for complement activation

As shown in figure 16 and figure 10, alternative pathway activation bypasses the antibody dependent steps that involve C1qrs, C4, and C2(55). This enables the alternative pathway to be active against infectious organisms prior to the development of specific antibody. The fundamental steps involved in alternative pathway activation are outlined in figure 17. Major activators of the system include endotoxic lipopolysaccharide from gram negative bacteria and techoic acid and peptidoglycans from some gram positive organisms. These initiating substances fix to key glycoproteins in plasma that include factor B, factor D, properdin, and C3b. Somehow, a protected C3b molecule must be formed to promote C3 cleavage in the alternative pathway. This C3b may come from leftovers from classical pathway activation, routine turnover, or by host or bacterial proteases. If C3b thus formed promptly attaches to an alternative pathway activator, it escapes

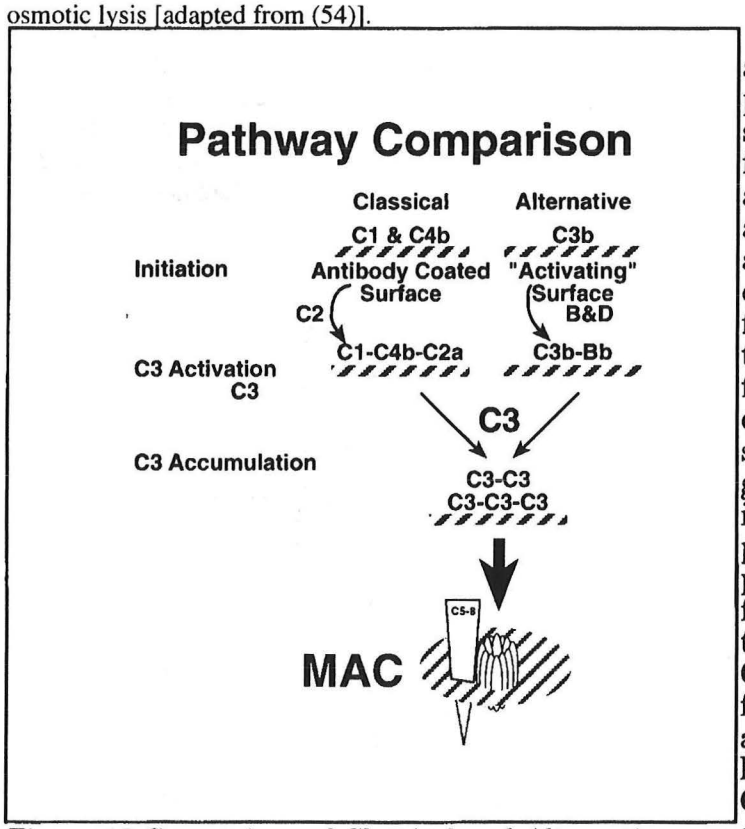

Figure 16-Comparison of Classical and Alternative Complement Activation Pathways [adapted from (55)]

inactivation and binds factor B (C3bB). Factor B can then catalyze activation of the complex to form activated C3bBb. This complex amplifies the hydrolysis of more C3. Polymers of this structure, $C3b_nBb'$ are stabilized by properdin and they activate C5 and the MAC. When C3b activated this way is inadvertently attached to host cells, it is rapidly inactivated by DAF-CD55 and other factors. Homologous restriction factor(HRF) and especially MIRL-CD59 interfere with formation of the MAC and therefore play key roles in the protection of host cells from innocent bystander damage induced by any complement-mediated process.

Alternative Pathway Activation Sequence

C3 cleavage via:
- **Classical pathway (infections)**
- **normal turnover**
- **Fibrinolysis (plasmin activation)**
- **Proteases (host or microbial)**

C3b to a site that protects it from inactivation by factor I and its' cofactors

C3b binds factor B to produce C3bB

Activated factor D activates the above complex to activated C3bBb

Activated C3bBb is stabilized by properdin

C3bnBb cleaves C5 in an amplification step that directly produces membrane attack complexes

Figure 17-Activation Sequence for the Alternative pathway of Complement [adapted from (54)].

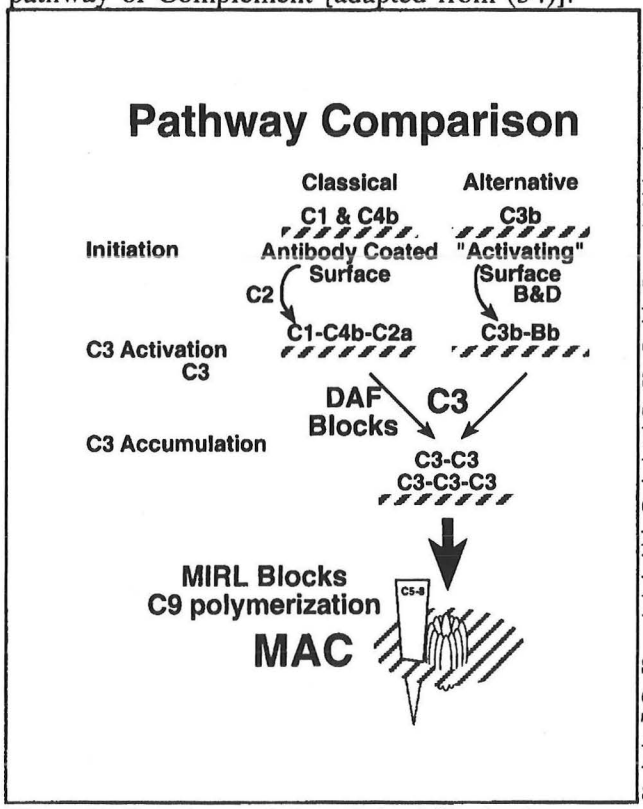

Figure 18-Complement is intended to kill microbes. Decay accelerating factor (DAF) and membrane inhibitor of reactive lysis (MIRL) are expressed on RBCs to prevent bystander damage. This cartoon shows where in the cascade, the GPI proteins intervene [adapted from (55)].

All three of these proteins, DAF(56), HRF(57), and MIRL(58), are deficient in PNH erythrocytes. DAF and MIRL seem to be the most important (see figure 18). The ability of DAF to impair C3b binding to erythrocytes was initially believed to explain complement sensitivity of PNH RBCs. Three distinct lines of evidence, however, established the preeminent role of MIRL deficiency in the pathogenesis of hemolysis in PNH.

First, Holguin et al showed in elegant experiments that complement-mediated lysis of PNH III cells could be completely prevented by adding solubilized MIRL to the defective erythrocytes(59) (see figure 19). Untreated PNH III cells lack DAF, HRF, and MIRL and the MIRL treated cells still lack DAF and HRF. These results strongly suggest that DAF and HRF play relatively minor roles in the pathogenesis of PNH.

This conclusion was bolstered by the same group in an elegant series of experiments using DAF deficient cells. They studied a patient from the Parkland Hospital blood bank who has an isolated deficiency of DAF and **no** evidence of clinical PNH. As expected, his RBCs suffered a 20-fold increase in C3 deposition in an acidified serum test but were not lysed(60). These cells have normal expression of MIRL. When the

RBCs were treated with antibodies that inactivate MIRL, the cells become sensitive to complement-mediated lysis in the Ham test.

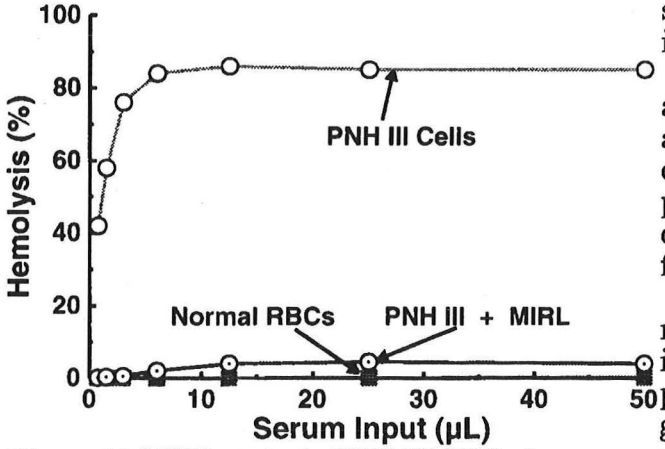

Figure 19-MIRL protects PNH III RBCs from complement-mediated lysis. RBCs were treated with cobra venom factor to activate complement. Normal RBCs (O) were not lysed. PNH III RBCs were nearly all lysed (■). If PNH III RBCs were pretreated with purified MIRL (⊙), lysis was prevented (58).

Third, a patient with an inherited and isolated deficiency of MIRL-CD59 also has PNH(61). Thus, it appears clear that while PNH RBCs lack many proteins, the hemolysis is primarily due to MIRL deficiency in cells derived from the diseased stem cell clone.

Besides their roles in complement modulation, the only feature they share is that they are all plasma membrane proteins anchored to cell surface by glycosyl-phosphatidylinositol. This single observation led to the discovery of the unique and fascinating molecular biology of PNH pathogenesis.

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired genetic disease of hematopoietic stem cells. The diseased cells descend from a single stem cell clone that has lost the ability to synthesize glycosyl-phosphatidylinositol (GPI) anchors required by a unique cadre of plasma membrane proteins. The loss of the GPI-anchoring system leads to most if not all of the clinical features of PNH. We will therefore review the process cells use to attach proteins to the cell membrane with GPI-anchors.

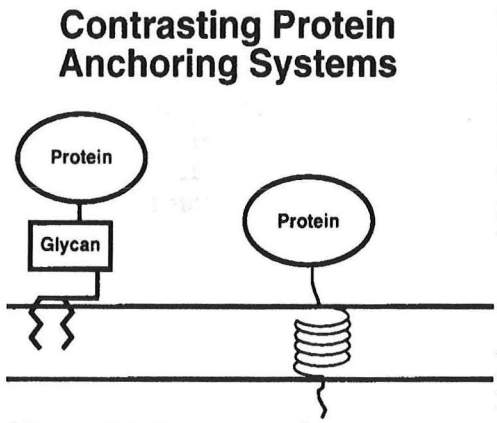

Figure 20-Protein Anchors-Most plasma membrane proteins have membrane spanning domains (right). The LDL-receptor is an example. GPI-anchored proteins (left) sugar phospholipid anchors with two fatty acid moieties in the outer leaflet. These anchors have been called glycosyl-phosphatidylinositol (GPI) or phosphatidylinositol-glycan (PIG-tails). The GPI anchor confers aunique abilities to cluster in caveolae (little caves).

B. GPI-anchoring system

Most membrane bound proteins possess hydrophobic amino acid domains that span the membrane. In the last decade, a novel method of anchoring plasma membrane proteins was discovered(reviewed by Low)(62) (see figure 20). This system features completely separate synthesis of proteins and anchors. The biosynthesis of GPI-anchored proteins has been reviewed extensively and recently(23, 63, 64). Investigators have discerned most of the steps in GPI biosynthesis by studying cell lines with defective GPI biosynthesis. The basic strategy is to mutagenize cells and select lines for homozygous defects in GPI synthesis. Cell lines are then classified by their ability and inability to correct GPI synthesis defects when fused to other mutant cell lines. The ability to correct defects in fusion experiments is called *complementation*. (I

apologize for the similarity to "hemolytic complement"-they are unrelated). Cell lines that can NOT complement each other are considered a complementation group. Lines from different complementation groups can complement each other in fusion experiments by definition. Traditionally, complementation groups are denoted by capital letters beginning with A. The complementation groups related to GPI biosynthesis are named PIG-A through PIG-H for phosphatidylinositol glycan(PIG tails), an alternate name for GPI.

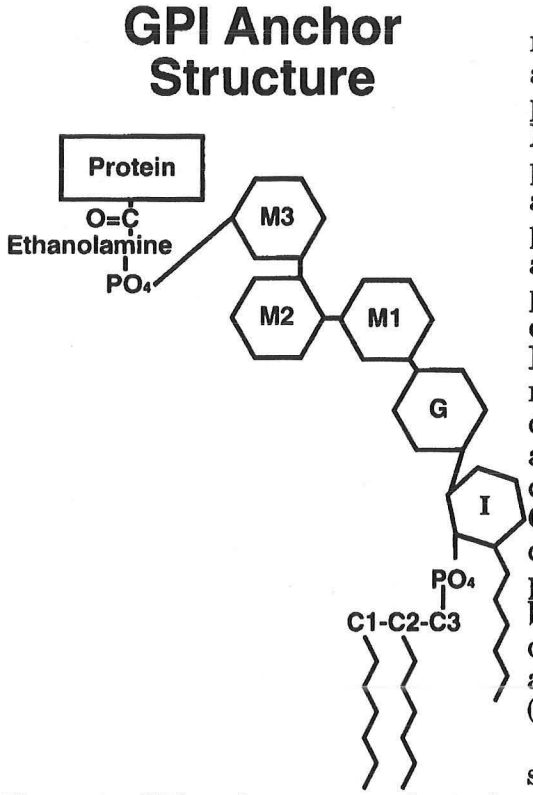

Figure 21-GPI anchors are synthesized by the sequential addition of inositol (I), glucosamine (G), three mannose residues (M1,M2,M3), and phosphoethanolamine. ER enzymes cleave GPI signals from nascent proteins and amidate the new carboxyl-terminus to GPI to complete the anchoring process. Mammalian cells remove the acyl chain from the inositol before the proteins reach the cell surface [adapted from (22)].

The first step in GPI biosynthesis appears to require the three proteins defined by PIG-A, C, and H complementation groups. These three proteins apparently conspire to add N-acetyl-glucosamine (GlcNAc) to phosphatidylinositol (PI) using UDP-GlcNAc as the sugar donor. The role of the PIG-C protein is not known but PIG-A and PIG-H appear to bring the substrates together with the proper geometry for the reaction but the site of catalytic activity has not been elucidated. The PIG-A protein appears to be a Type II membrane protein with the amino-terminus dangling in the cytoplasm. PIG-H also appears to not be a luminal ER protein consistent with the knowledge that the GlcNAc-PI resides primarily on the cytoplasmic side of the ER membrane(65). This poses a potentially large problem for the cell because the completed GPI moiety must reside on the luminal side of the ER membrane to be attached to nascent proteins during synthesis. (see figure 21).

The molecule then undergoes a series of steps. These include deacetylation to form glucosamine-PI. Acyl-CoA then donates a fatty acid to the inositol conferring resistance to PI-PLC but not PI-PLD. Somewhere near this step, the molecules "flips" to the luminal leaflet of the ER membrane. The exact timing and mechanism are still mysteries. Next, three mannose residues are added sequentially with dolichol-phosphate-mannose (DPM) as the mannose donor(66). Phosphatidylethanolamine is the donor for phosphoethanolamine addition to the third mannose residue. In mammalian GPI, ethanolamine is usually added to the other two mannose residues as well but they do not participate directly in attachment of the protein. Cell surface sensitivity to PI-PLC is restored by removal of the acyl group esterified to the inositol moiety. This mature preformed structure is ultimately attached to a specific subset of nascent proteins synthesized on ER ribosomes. This completes the first of the two converging biosynthetic pathways involved in the synthesis of GPI-anchored proteins. The first step in GPI-anchor synthesis is

defective in PNH affected cells and all the cases described to date have defects in the mutant gene corresponding to the PIG-A complementation group(21, 67-70) and none to groups PIG-C or PIG-H.

GPI-anchored proteins share cleavable hydrophobic domains at their C-termini. These domains signal attachment of preformed GPI-anchors. GPI-anchors are covalently bound to the new C-terminus of the nascent proteins formed by cleavage of the hydrophobic signal(71, 72). Synthesis of GPI-anchored proteins is the result of merging two biochemical pathways. The first is GPI-anchor synthesis described above and the second is the endoplasmic reticulum (ER) based, synthetic machinery for secretory proteins. This requires an amino-terminal hydrophobic signal sequence that directs ribosomal protein synthesis to the lumen of the ER. Generally, signal sequences are cleaved co-translationally by signal peptidase. GPI-anchored proteins have a different signal at the carboxyl-terminus. After cleavage of the C-terminal signal peptide from the proprotein, a putative transamidase forms an amide bond between the new C-terminus and the amino group of the terminal ethanolamine in GPI. Several lines of evidence showed that biosynthesis of anchors and proteins as well as their covalent attachment occurs in the ER. First, the cDNA for every GPI protein described to date contains a consensus amino-terminal signal peptide directing protein synthesis to the ER. Second, proteins with uncleavable carboxyl-terminal signals are trapped in the ER(73, 74). Third, brefeldin A treatment has no effect on GPI-anchor attachment in spite of its' ability to disaggregate the Golgi(75). This proved that GPI-anchoring is complete before proteins reach the Golgi leaving the ER as the only possible site for the process to occur.

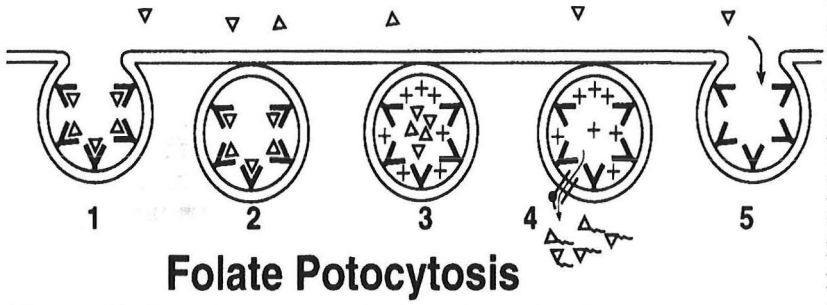

Figure 22-Caveolae cluster GPI-anchored folate receptors up to 800 receptors per pit where they bind 5-MeTHF with high affinity(1). Caveolae close sequestering ligand-receptor complexes(2). Acidification of the caveolar lumen releases the bound folate (3) an generates and estimated 100,000 fold increase in concentration. The reduced folate carrier (4) regulates folate entry prior to recycling of caveolae (5).

GPI-anchored proteins have no amino acids interacting with the membrane but instead are anchored by two fatty acid moieties dangling in the outer leaflet of the membrane. This contributes to greater mobility in the plasma membrane. GPI-anchors confer "spelunking" on preteins by providing targeting signals directing cells to place these proteins in caveolae(latin for *little caves*). Caveolae are lipid-defined patches of plasma membrane enriched in multiple proteins that specialize in signaling and nutrient uptake (see figure 22). The spectrum of tasks subserved by GPI-anchored proteins is broad and therefore a cellular loss of GPI-anchoring causes many different deficiencies that were heretofore apparently unrelated.

C. Defect in GPI-anchoring

In most PNH patients, erythrocytes, leukocytes, and platelets derived from the PNH clone are completely deficient in GPI-anchored proteins (PNH III

phenotype) while the remaining cells are completely normal. Fourteen GPI-linked proteins are known to be deficient in PNH cells and they are listed in figure 23. These proteins have normal functions ranging from complement defense (DAF-CD55, MIRL-CD59, and C8 binding protein-homologous restriction factor-HRF), surface enzymes, and receptors, to proteins with of unknown function. No doubt there are multiple other GPI-proteins yet to be discovered that play roles in the pathogenesis of PNH.

While there are many steps in GPI-anchor biosynthesis, deficiency of only one accounts for essentially all of the PNH cases that have undergone genetic characterization(21, 67-70). The defective gene in PNH is called PIG-A because it is also the defective gene in the PIG-A complementation group of cells incapable of synthesizing GPI-anchors. The PIG-A gene product along with the products of PIG-C and PIG-H are responsible for the first step in GPI synthesis, the addition of N-acetyl-glucosamine (GlcNAc) to phosphatidylinositol on the cytoplasmic leaflet of the ER membrane. PNH has never been described with defects in PIG-C and PIG-H even though mutants developed in vitro have essentially identical phenotypes. It was also somewhat surprising that no cases of PNH were seen with other defects in GPI biosynthesis because again the somatic mutants all exhibit defective GPI-anchor biosynthesis. All of these defects cause proteins normally destined to have GPI-anchors to be synthesized without an anchor and therefore fail completely to traverse the secretory pathway to the plasma membrane.

Proteins Deficient on PNH III Cells

- **Complement Defense Proteins**
  - DAF (CD55
  - MIRL (CD59)
  - HRF (C8 binding protein)
- **Enzymes**
  - Acetylcholinesterase (RBC)
  - Alkaline phosphatase (leukocyte)
  - 5' nucleotidase (lymphocyte\)
- **Receptors**
  - Fcγ receptor III (CD16a-phagocytic)
  - Folate receptor (RBC progenitors)
  - Urokinase receptor
  - LPS binding protein receptor (CD14)
  - Immunological contact receptors
    - LFA-3 (CD58) (all cells)
    - CD48 (lymphocyte)
    - CDw52 (lymphs monocytes)
- **Other proteins of unknown function**
  - JMH-bearing protein (RBC)
  - CD24
  - CD66
  - CD67
  - p-50-80 (granulocyte)

Figure 23-PNH III cells lack these GPI-anchored proteins [adapted from (22)].

D. Chromosomal localization of PIG-A explains the unique genetic biology of PNH

Recessive genetic traits are not commonly acquired because a double hit is so rare. One of the major exceptions is a class of inherited heterozygous defects in tumor suppressor genes. Cells with one defective allele are phenotypically normal but now a single hit on the only normal allele creates a phenotype capable of uncontrolled growth producing disease states such as adenomatous polyposis coli and childhood retinoblastoma. This sort of mechanism was never seriously considered in PNH because the disease is not inherited. The fact that our case had a brother with PNH is very unusual. Why then would we expect to see an acquired disease with a total loss of GPI-anchor synthesis. The answer to this question unexpectedly also explained why is PNH not seen with other defects in GPI-anchor synthesis besides PIG-A. The PIG-A cDNA was cloned and its structural gene mapped to the short arm of the X-chromosome in 1993 (21, 76). This observation was not anticipated but provided a prompt and sensible explanation for the unusual genetic biology of PNH. Males have only one

X-chromosome so a single hit on their only PIG-A allele eliminates GPI-anchor synthesis. During development, cells in females "lyonize" or inactivate one of their two X-chromosomes. While the initial lyonization event in any given primordial cell is functionally random, daughter cells keep the same X-chromosome lyonized throughout life. If a hematopoietic stem cell suffers a "hit" on its' only functioning PIG-A gene, it will reliably pass on the defect to all line precursors and progeny. All humans (and mice) are functionally hemizygous for PIG-A and therefore at risk of a single hit inducing the disease.

This discovery also explains the probable reason that no other defects in GPI biosynthesis have been shown to cause PNH. We suspect that all of the other genes reside on autosomes and are therefore not at risk in single hit events. PIG-H is known to be on chromosome 14(77) and PIG-F is on chromosome 2(78).

Since these data show that PNH arises as a spontaneous mutation in a stem cell, we would expect PIG-A mutations to differ from patient to patient and this is indeed the case. Most imaginable variants of mutations have now been described(21, 69, 70). Some patients missplice nuclear RNA for PIG-A(21). Others have deletions of a one or two nucleotides in a coding region causing a frame shift and premature stop codon. Multiple missense mutants have been described but the molecular defects are unknown because there is no assay for the function of the PIG-A gene product.

A more detailed understanding of the gene defects and their effects on the protein should also help unravel the reason(s) for existence of so-called PNH II cells(10). PNH II cells exhibit complement sensitivity that is 3-5 times greater than normal cells (PNH I) but 3-5 fold less than completely defective PNH III cells. Genetic defects that might be anticipated to produce PNH II cells include promoter defects and reduce synthesis of a normal PIG-A mRNA, mutations that reduce mRNA stability, and missense that decrease without eliminating activity of the PIG-A protein. Curiously, PNH II defects not only produce intermediate effects on complement sensitivity of erythrocytes, but different GPI-linked proteins suffer differential impairment. For example, cells from some PNH II patients express relatively more FcγIII receptor than CD55 and CD59(79). No clear biochemical explanation for this phenomenon has been determined but it suggests that some GPI-linked proteins are better substrates for anchor attachment and that they out compete other proteins for the relatively scarce anchor substrate.

E. Mechanisms of increased thrombosis

PNH platelets exhibit increased membrane vesiculation induced by the membrane attack complex (C5b-9). The MAC causes normal platelets to cluster and pinch off the MACs in vesicles. These vesicle expose acidic phospholipids and serve as a site for the formation of the prothrombinase complex by factors V, VIII, and X(80). Sims et al showed that antibodies against MIRL-CD59 dramatically enhance vesicle formation by otherwise normal platelets(81). PNH platelets have no MIRL suggesting the possibility that even minimal complement activation may increase vesiculation leading to thrombosis. While this is the best current theory for explaining thrombosis in PNH, the true cause *in vivo* remains to be established.

Finally, peripheral blood mononuclear cells normally express a receptor for urokinase-type plasminogen activator (uPA-R). This receptor is anchored by GPI(82) and bound uPA activates plasmin. It is believed that uPA-R positions uPA activity controlling precisely the site(s) of plasmin generation. Since

plasmin is the critical enzyme in degrading clots, the inability of white blood cells to target plasmin generation by this normal mechanism may contribute to thrombotic events in PNH.

Peripheral Blood BFU-Es in PNH

	No. of Subjects	Erythroid Colonies
Normal subjects	12	20.1 ± 1.9
PNH patients	7	2.8 ± 0.6

Figure 24-Burst forming units-erythroid (BFU-E) can be isolated from peripheral blood. Their numbers are markedly reduce in PNH patients(25).

PNH Cell Life Span

PNH I	120 days
PNH II	45 days
PNH III	8 days

Figure 25-RBC life span by PNH cell type. PNH I cells are normal, PNH III are severely affected by PNH, and PNH II cells are exhibit intermediate complement sensitivity and have an intermediate life span.

F. Proposed biology of aplastic anemia in PNH

Many investigators now believe that all patients diagnosed with PNH have a component of aplastic anemia. There are multiple reasons for this opinion. First, PNH patients have significantly fewer burst forming units-erythroid (BFU-E) circulating in peripheral blood than normals (see figure 24). The second is that the normal contribution of any single stem cell is far less than the 1% threshold required to detect PNH. If the normal marrow stem cells produce at normal rates, the PNH clone could never produce 40% of circulating RBCs with 1/12th the normal life span (see figure 25 and 7). The juxtaposition of relative or absolute marrow hypoplasia with PNH suggests that either: 1) A single defective stem cell (PNH clone) takes over the marrow causing the normal stem cells to function poorly or 2) Marrow hypoplasia (say immune mediated) produces an environment favorable for selection of a clone that can escape the immune mediated attack that depletes the normal stem cells(83). The latter hypothesis is gaining credibility because of the nearly universal hypoplasia in PNH, the common history of aplastic anemia preceding and benefitting from the development of PNH, and the therapeutic responses to marrow transplantation in patients without marrow ablation(84, 85). Some have suggested therefore, that aplastic anemia provides an ideal environment for the selection of a PNH clone resistant to whatever causes diminished proliferation of normal stem cells in aplastic anemia. This is supported by a case report of antilymphocyte globulin therapy reversing both aplastic anemia and PNH in a single patient(86). Third, recent studies suggest that a higher proportion of aplastic anemia patients have PNH clones than was previously believed(87). Finally, there are multiple reports of marrow transplantation reversing aplasia and the PNH even without marrow ablative therapy preceding the transplant(84, 88, 89). I believe the best hypothesis is now that stem cell depletion (by one of several possible mechanisms) is permissive for development of PNH(26, 85) (see top of figure 9).

G. Proposed Mechanisms for increased infection in PNH

The cause(s) have not been identified but is no doubt related to the loss of one or more GPI-anchored proteins. While multiple immune modulators have GPI-anchors, the best candidate for explaining increased infection in PNH is the Fcγ receptor III. Fcγ receptors I and II have transmembrane anchors while Fcγ receptor III has a GPI-anchor and is the major Fc receptor on human granulocytes. FcγR I, II, and III are all believed to facilitate phagocytosis of microbes coated with antibody. The presence of FcγR I and II apparently diminishes the effect of losing FcγR III in PNH granulocytes but in some cases the compensation may be inadequate leading to increases in bacterial infections. Other immunologically relevant lymphocyte surface proteins have GPI-anchors but their role in PNH infections has not been established nor have mechanisms been proposed. The most interesting example is CD14. CD14 has a GPI-anchor and is the receptor for complexes of endotoxin lipopolysaccharide(LPS) and the LPS binding protein. The diminished expression of CD14 on PNH monocytes and endothelial cells may alter clearance of bacterial endotoxin which could play role in the increased susceptibility to infection(90, 91).

MIRL-CD59 also plays a role in T-cell rosette formation and T-cell activation. Recent studies indicate that MIRL is involved in antigen presentation(92).

No single observation explains infection susceptibility in PNH but multiple GPI-anchored proteins subserve roles in defense against infection and we anticipate that a more coherent story will emerge in the coming years.

V. **Differential Diagnosis of PNH**

A. Erythrocyte production and clearance

The average adult human has about 10^{12} progenitor cells in the hematopoietic system. These cells are confined in approximately 1.7L of marrow. The marrow serves much more than a housing function for these cells because it provides the microenvironment on which efficient and effective hematopoiesis so heavily depends. The process of producing all the various lines of marrow derived cells is complex but so tightly regulated that steady-state levels of most lineages are nearly constant. The pluripotent stem cell (CFU-S: colony forming unit-spleen) is the progenitor of all the various cell types from RBCs to eosinophils to platelets and probably even lymphocytes (see figure 26). The average adult appears to have between 10^6 and 10^7 pluripotent stem cells so they represent only 0.0001% to 0.001% of the cells in the marrow. This 1 to 10 mg of stem cells is an extraordinarily precious commodity that can only be replaced by transplantation. Stem cell replacement by transplantation requires only about 5% of the original number extant in a healthy marrow.

Pluripotent stem cells divide rapidly and must "choose" to either differentiate into a progenitor cell for one of the specific pathways or replenish stem cells lost to differentiation (see figure 26). If the renewal process falls behind losses to differentiation reducing the total stem cell pool, aplastic anemia may result. Most marrow derived cells originally differentiated from a stem cell (CFU-S) to a multipotent stem cell (CFU-GEMM) capable of becoming a progenitor of erythroid, granulocytic, macrophage, eosinophilic, or megakaryocytic progenitors. Many of the molecular regulators of differentiation and development have been identified, cloned, and several are now available for clinical use. The most primitive dedicated erythroid precursor (BFU-E) is marginally sensitive to

erythropoietin. Peripheral BFU--Es are dramatically reduced in PNH (see figure 24). The next progenitor (CFU-E), has significantly increased erythropoietin receptors and is much more sensitive. Commitment to RBC development places enormous demands on cells for iron acquisition. Erythropoietin stimulates synthesis of transferrin receptors to facilitate iron uptake via clathrin-coated pits in erythrocyte precursors. The correlation between transferrin receptor synthesis and erythropoietic activity has become the basis of an assay that enables clinicians to distinguish clearly between anemia of chronic disease and iron deficiency without invasive or complex kinetic measurements. Anemia of chronic disease is not associated with increased circulating transferrin receptors but since iron deficiency stimulates the biology of erythropoiesis, circulating transferrin receptors increase iron deficiency anemia.

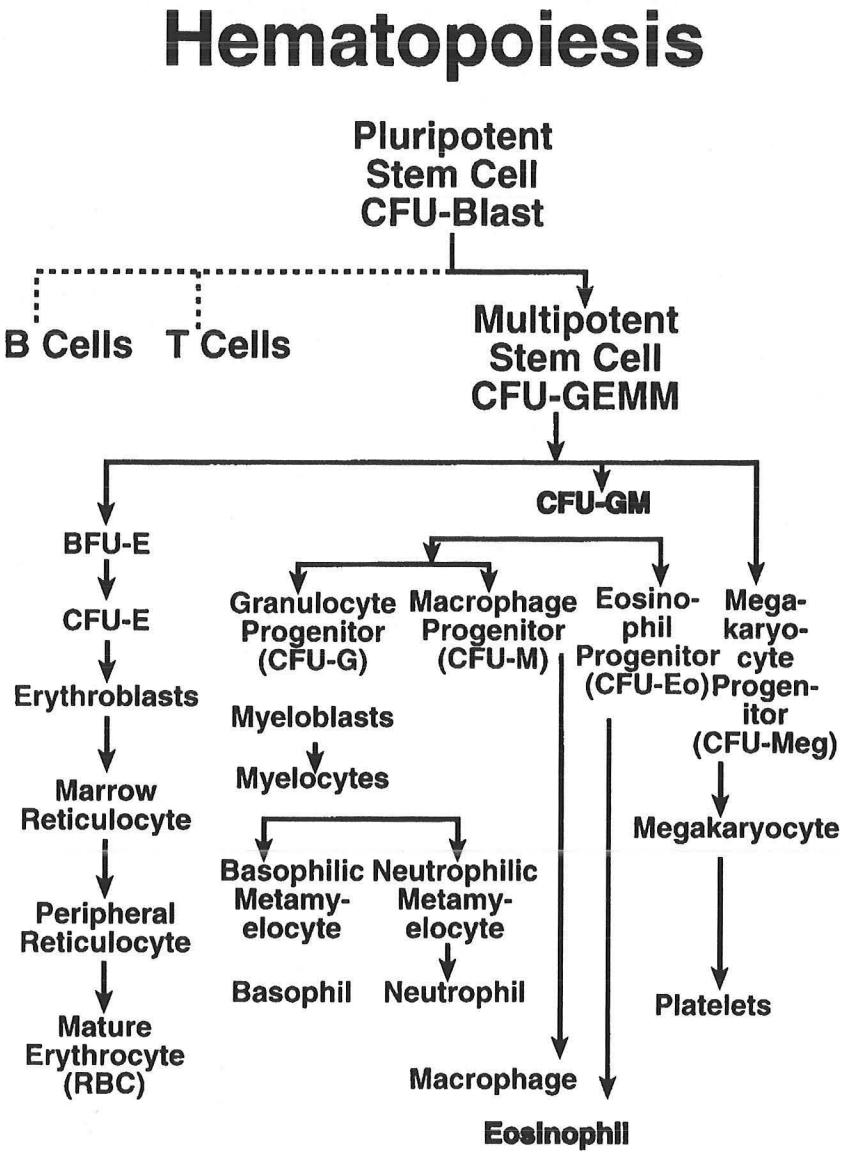

Figure 26-Hemopoiesis and Cell lineages-The developmental lineages of marrow derived cells are summarized in this cartoon [adapted from Scientific American Medicine ch. 5-I, p3., Stanley Schrier 10/94].

Reticulocytes are the result of nuclear extrusion by late erythroblasts. Reticulocytes are recognizable because they have no nucleus but they still have polyribosomes synthesizing hemoglobin. The life span of reticulocytes is four days and under normal circumstances, these cells spend their first three days in the marrow. The proportion of reticulocyte life span spent in the circulation (nl=25%) varies with disease and is a major source of confusion when clinicians attempt to interpret "reticulocyte counts." (Resolution of this confusion under "Evaluation of

Anemia".)

Bone marrow biopsy and aspiration remain invaluable tools in the assessment of anemia and pancytopenia. Prussian blue staining of marrow biopsies is the gold standard for determining hematopoietically relevant iron stores. Serum measurements of iron and iron-related proteins for practical purposes have supplanted marrow examination but the unlike former, the marrow examination is practically void of false positives and negatives.

Erythrocyte function is dominated by oxygen delivery to peripheral tissues. 100 ml of blood can contain up to 20 ml of oxygen bound to 15 gm of hemoglobin. Inadequate delivery of oxygen to peripheral tissues stimulates an unknown cell in the kidney to produce erythropoietin. Erythropoietin stimulates RBC production with its attendant increase in transferrin receptor synthesis(93) leading to reticulocytosis. When anemia supervenes, one of more of these functions is abrogated and direct and indirect measurement of these phenomena provides the modern basis for the diagnosis of anemia(94).

B. Evaluation of Anemia

1. Introduction

Anemia does not have a straightforward definition based on the result of a single laboratory test. Pregnant women in the third trimester experience a one liter expansion of plasma volume with a concomitant increase in RBC mass of 250 ml. Thus, the hematocrit falls but clearly does not indicate anemia. Conversely, bleeding from trauma or the GI tract can cause an acute one liter decrease in blood volume that produces shock with no change in the hematocrit at all. Some hemoglobinopathies (Hgb-Kansas) enhance oxygen unloading allowing low hemoglobin levels (8 gm/dl) to meet physiologic oxygen demands perfectly. Clinicians must combine clinical judgment with laboratory evaluation of morphology and kinetics to arrive at the diagnosis of anemia and to discern its cause in individual cases.

Anemias can be divided quickly into three cause groups: **blood loss**, **production defects**, and **hemolysis**. Most anemia due to blood loss is caused by gastrointestinal or gynecologic losses. Hospitalized patients may lose up to 300 ml of blood per week from routine blood draws for laboratory tests.

2. Blood loss

When following a patient with a falling hematocrit, a simple calculation will allow one to determine if defective production can explain the anemia or whether blood loss is required to explain the fall. Zero RBC production causes a 1% decrease in RBC number per day because RBCs have a ~100 day life span. Rates of fall of greater 1% per day other explanations. For example, a fall in hematocrit from 40 to 30 over one week represents a 25% decrease in RBCs or more than 3% per day indicating blood loss or hemolysis.

3. RBC production

While sophisticated and highly accurate methods exist for measuring RBC production, a small set of tests properly interpreted can closely approximate true RBC production. These include the hematocrit, RBC count, reticulocyte count, and one of two different methods for "correcting" the reticulocyte count. The most precise correction scheme is called the corrected absolute reticulocyte count. Normally, reticulocyte "counts" actually represent the percentage of RBCs that are reticulocytes. This is no more a "count" than when a

differential reports 70% neutrophils. A true count depends on multiplying the percent reticulocytes by the total RBC count. Normal absolute reticulocyte counts are between 25,000 and 75,000. Even absolute reticulocyte counts though are inadequate because they fail to account for alterations in reticulocyte biology induced by anemia. Reticulocyte life span is 4 days with only the last full day spent in the circulation of non-anemic patients (see figure 27). As anemia worsens, however, the proportion of the 4 days spent in the circulation increases from 1 day (Hct 40-45) to 2.5 days (Hct 15). The corrected absolute reticulocyte count is calculated by dividing the absolute count by the maturation time in days (see figure 28). Consider two patients with hematocrits of 15 (RBC count 1.67 x 10^6) and 45 (RBC count of 5 x 10^6) and classical reticulocyte counts of 4% and 1%. The corrected reticulocyte count for the anemic patient is [1.67 x 10^6 x (0.04)] / 2.5 (days) = 26,720 while the normal patients' corrected absolute reticulocyte count is [5.0 x 10^6 x (0.01)] / 1.0 (days) = 50,000. The normal patient has a count at the midpoint of the normal range. The anemic patient is producing RBCs at a rate just inside the normal range on the low end but would have elevated RBC production were it not for the marrow contributing to the problem. This kind of observation is often missed because most clinicians instinctively view a reticulocyte count of 4% as an adequate response to anemia. Clearly, RBC production must be assessed in a more comprehensive way than simply measuring the reticulocyte count. When true RBC production fails to increase in anemia the hematopoietic defect represents all or part of the problem and reasons must be sought. Consider our patients' initial presentation. Her reticulocyte counts ranged from 2.7% to a high of 5% with RBC counts and hematocrits typically about 2.4 x 10^6 and 24 respectively. Her corrected absolute reticulocyte counts ranged from about to 32,400 to 59,000 with the lower more common than the higher. Clearly, her RBC production rate is VERY low for one

Reticulocyte Maturation Time (Days)

Hematocrit (%)	Maturation Time (days)
45	1.0
35	1.5
25	2.0
15	2.5

Figure 27-Reticulocyte Maturation Time is the time (in days) required for reticulocytes released from the marrow to fully mature in the circulation [adapted from the Red Cell Manual, Hillman and Finch]

Corrected Absolute Reticulocyte Count

		Example Our Case
Absolute Reticulocyte Count	= RBC Count X Reticulocyte Count (as fraction)	2,400,000 X 0.027 = 64,800
Corrected Absolute Reticulocyte Count	= Absolute Reticulocyte Count / Reticulocyte Maturation Time (days)	64,800 / 2.0 = 32,400

Normal = 50,000±25,000 must be interpreted

Figure 28-Calculation of the corrected absolute reticulocyte count-The calculation is performed as shown. Our patients' corrected count was 32,400. This is a normal count in non-anemic patients. In an anemic patient, this count is low and demonstrates that the anemia is partly due to a RBC production defect.

so anemic.

Another method of estimating RBC production is a little more intuitive and only slightly less accurate called the reticulocyte production index. This method yields numbers comparable to the standard reticulocyte count making interpretation easier for those who only need to perform such assessments occasionally (see figure 29). The reticulocyte count is multiplied by the ratio of hematocrit to a normal hematocrit and then divided my reticulocyte maturation time in days. A normal reticulocyte production index is 1. It will tend to increase if marrow responses to anemia are normal and decrease if marrow defects contribute to the anemia. Both methods of assessing RBC production are useful clinically and one of the two must be used to properly interpret reticulocyte counts. When low RBC production is confirmed, the diagnosis can be made by following the algorithm shown in figure 30.

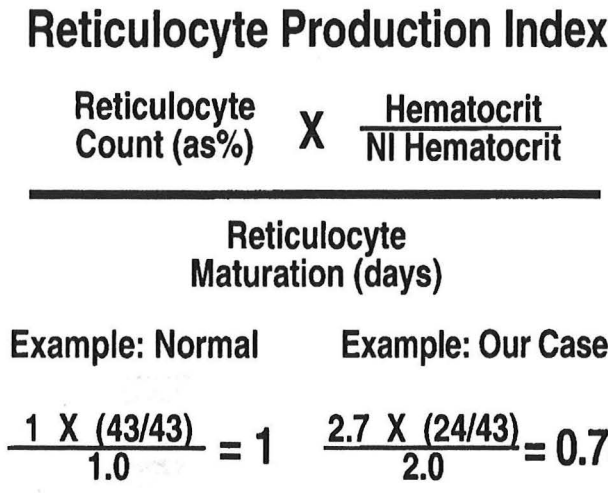

Figure 29-Reticulocyte Production Index- Calculation is performed as shown. It corrects for both hematocrit and reticulocyte maturation time [adapted from the Red Cell Manual, Hillman and Finch]. Extravascular hemolytic anemias usually have an index over 3 with 1 being a normal index. Our patients' low index confirms her severe RBC production defect.

4. Evaluation of the peripheral blood smear

The JCAH notwithstanding, there is no substitute for examination of the peripheral smear. Nothing helps the clinician visualize the biology of the patient's anemia like the microscopic appearance of the blood. RBC morphology, platelet clumping, segmentation of neutrophil nuclei, subtle hypochromia, basophilic stippling, and fragmenting of RBCs can only be appreciated by visual examination and this author laments current policies that impair the routine passage of these skills to current and subsequent generations of physicians.

5. Iron metabolism: evaluation and deficiency

Iron deficiency is usually present in PNH but its pathogenesis is different from the usual case of iron deficiency. Iron metabolism is quite unusual because the body has no regulated mechanism for ridding itself of excess iron. This is presumably due to iron excess being too rare in the ancestral environment to establish an advantage for development an iron elimination system. Iron metabolism is regulated entirely by controlling absorption from the GI tract. Under the best of circumstances, only 35% of ingested heme iron can be absorbed and only 5% of non-heme iron is available for absorption. Non-heme iron is more bioavailable if it is reduced from Fe^{3+} to Fe^{2+}. Vitamin C (ascorbic acid) improves iron absorption because it reduces Fe^{3+} to Fe^{2+}. The ability of pH<3 to similarly reduce iron explains which patients with vagotomy and antrectomy are at greater risk for iron deficiency. Typically, enterocytes of the duodenum and proximal jejunum absorb 12% of dietary iron and only transfer ~3.5% of dietary load to the

bloodstream before they are sloughed. In iron deficiency, the percent absorbed increases to 33% and almost all of it is delivered to the bloodstream. Patients with hemochromatosis absorb iron at just slightly less than rate of patients with iron deficiency.

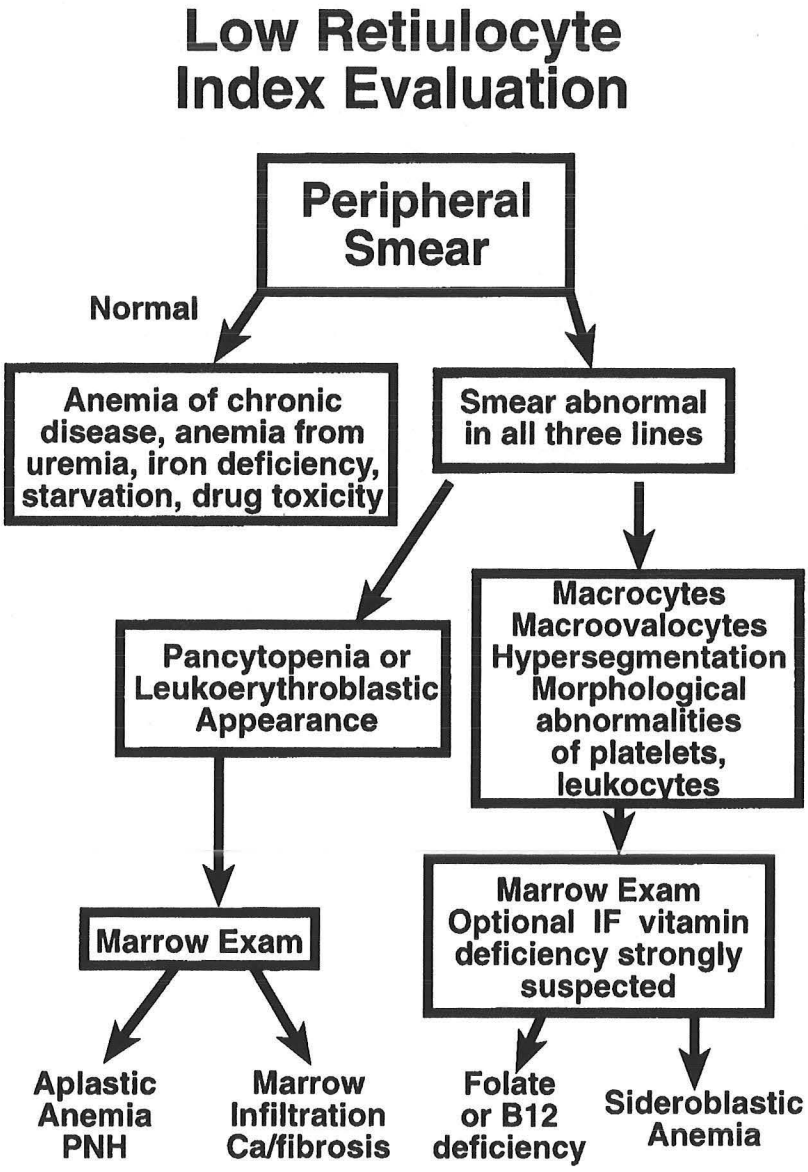

Figure 30-Algorithm for evaluation of anemia with decreased RBC production [adapted from Scientific American Medicine ch. 5-III, p2., Stanley Schrier 6/89].

Iron balance in women is tenuous because typical iron absorption is about 1-2 mg per day and women lose 1 mg per day in skin, nails, urine, and stool and another 1 mg per day (average) due to menses. This fragile balance is severely stressed by pregnancy in which an additional 800 mg of iron is lost per child. These losses are difficult for women to replace. Men lose only 1 mg per day and dietary uptake can easily average 2-2.5 mg per day if necessary. Thus, iron deficiency is rarely dietary in men but can easily be partly or totally dietary in women. Most of the iron flux in plasma (80%) is harvested from dying RBCs by macrophages and recycled back to the marrow bound to transferrin to support erythropoiesis. This system has a large capacity explaining why so-called extravascular hemolysis generally does NOT produce iron deficiency. In extravascular hemolysis, the iron from cleared RBCs is available for hemoglobin synthesis within minutes. This is why RBC production can increase 7-fold in hemolysis in contrast to blood loss. When blood is lost, iron must be mobilized from storage forms, ferritin and hemosiderin, so that a 70% increase in hemoglobin is all the marrow can accomplish.

One of the most unusual causes of iron deficiency is PNH. PNH is unusual because most hemolysis in other diseases is extravascular where iron recycling is highly efficient. The hemolysis in PNH, however, is almost entirely intravascular. There is no good mechanism for reclaiming iron lost to

intravascular hemolysis in the amounts produced in PNH. Some of it is reclaimed by renal proximal tubule cells but much of it is lost as hemosiderin in sloughed epithelial cells.

Iron circulates bound to transferrin and is cleared mostly by RBC precursors using the transferrin receptor. This receptor enters the cell via clathrin-coated pits and is recycled to the cell many times to repeat the cycle. Stimulation of erythropoiesis increases the number and cells in the marrow bearing transferrin receptors and secondarily circulating transferrin receptors increase under these conditions. This observation is becoming important the assessment of difficult anemias.

Since circulating ferritin sequesters iron from cells, it tends to fall in iron deficiency and rise in iron overload. Ferritin is usually low (20-100) in anemia of chronic disease and thalassemia trait. Ferritin can also be misleadingly high because it is an acute phase reactant. Virtually all cells make ferritin because free iron is highly toxic to living cells. Ferritin is large protein (480 kD) that can bind up to 4500 iron atoms each. Crystalline clumps of ferritin are visible on staining with Prussian blue as hemosiderin.

Iron deficiency states cause stimulation of erythropoiesis and since there is not enough iron to support it, cells have less than normal of hemoglobin and are smaller accounting for the typically low MCV. The stimulation of erythropoiesis increases transferrin receptor synthesis and release into the blood enabling us to readily distinguish iron deficiency and anemia of chronic disease which characterized by a production defect(95-97). The measurement of ferritin has become standard practice in attempts to rule in or out the diagnosis of iron deficiency. Interpretations must be cautious because ferritin levels fluctuate in response to processes unrelated to iron metabolism. In one study, the ferritin was not helpful in diagnosing 93 patients who actually had iron deficiency(98). Inflammatory diseases tend to increase ferritin levels as part of the acute phase response. One study in patients with rheumatoid arthritis examined various tests for their ability to predict responses to oral iron therapy. The best predictor was MCV < 80 fl/cell and ferritin < 110 ng/ml(99). In the absence of inflammation, many would consider a ferritin of 90 to be "proof" that iron deficiency is not present. When iron deficiency is suspected or suggested, especially in women, clinical trials of iron therapy should be strongly considered as the gold standard (marrow biopsy) is too invasive to be considered routine in the diagnosis of iron deficiency.

6. Iron replacement

Most PNH patients require iron replacement therapy because of prodigious urinary iron losses. Oral iron replacement should always use a reduced salt such as $FeSO_4$ and should never be enteric coated. Enteric coating delivers a significant fraction of the iron distal to the major site of iron absorption, the duodenum. GI symptoms develop so commonly in patient acutely started on 300 mg/day of $FeSO_4$ that the dose should be gradually increased to this level over about 3 weeks. Rarely, oral iron therapy is inadequate or not tolerated and parenteral iron therapy is required. (see figure 31) for calculation of total iron deficit and replacement by the parenteral route.

7. Folate deficiency

Folate deficiency is a near universal feature hemolytic diseases but may occur in many conditions (see figure 32). Folate circulates as 5-

methyl-tetrahydrofolate(5-MeTHF) free in the circulation. Most cells do not have specific folate receptors and the folate transporter has a low affinity for the vitamin. Thus, tissue cells do not efficiently reclaim folate released by hemolysis. With a molecular weight of approximately 440 daltons, 5-MeTHF is readily filtered by the glomeruli. Daily filtration exceeds 13 times total extracellular folate stores so that efficient reabsorption by the proximal tubule is required to prevent folate deficiency. This process depends on the GPI-anchored folate receptor(100) and potocytosis that reclaims 5-MeTHF from the glomerular filtrate(101, 102). While this system efficiently reabsorbs 5-MeTHF produced by normal RBC turnover, it is easy to imagine that it could be overwhelmed in PNH but this has not been studied. Presumed urinary losses of 5-MeTHF in the urine and extraordinary folate requirements make folate deficiency a near universal feature of hemolytic anemias. This phenomenon along with iron deficiency accounts for the "normal" MCV so typically seen in PNH as in our patient. Patients are usually deficient in both folate and iron so some cells tend toward macrocytosis (folate deficient) and others tend toward microcytosis (iron deficient). The MCV is therefore often normal as in our case (92) with a high RDW (24).

Parenteral Iron Dosage Calculation

60 kg woman with Hgb of 6 gm/dl.
What is appropriate parenteral iron dose?

Calculate Hgb deficit 14 - 6 = 8 gm/dl
8 gm/dl X 60 kg X 65 ml/kg = 312 g

Iron Deficit
312 g Hgb X 3.4 mg Fe/g Hgb = 1,060

Iron dextran at 50 mg Fe/ml given in 2 ml doses
Several doses a week is appropriate
1,060 mg/50 mg/ml = 22 ml or 11 doses

Figure 31-Parenteral correction of Iron Deficiency [adapted from Scientific American Medicine ch. 5-II, p10., Stanley Schrier 91]

C. Hemolytic Anemias

1. General comments on RBCs related to hemolysis

The normal RBC is shaped like a disk dented on both sides with a diameter of 7-8 μm which is about the diameter of a nucleus. In contrast to spherical nuclei, the flatter shape gives RBCs a high surface to volume ratio imparting the flexibility required for a cell to traverse capillaries with a diameter less than 6 μm. The average volume of an RBC (MCV) is between 85 and 90 femtoliters (μm^3, fL, 10^{-15} L) while the average surface area is 140 μm^2. Developing erythrocytes focus their energies on synthesizing hemoglobin which is supported producing mRNA for transferrin receptors and hemoglobin. Maturing reticulocytes lose their transferrin receptors but retain two fundamental groups of proteins defined by their anchors. The transmembrane proteins account for most of the polymorphic blood group antigens that are so critical to safe transfusion. The GPI-anchored proteins are critical for defense against attacks from host-derived complement.

RBCs are not cleared at random from the circulation. As they age, membrane is lost faster than intracellular volume. This decrease in the surface to volume ratio makes RBCs more stiff as does the concomitant increase in MCHC. Macrophages sense the progress of these changes and clear senescent RBCs.

2. Hemolysis

The degree anemia in hemolytic processes is a balance between the rate of RBC destruction and the extent of compensatory erythropoiesis. Five to seven fold increases in RBC production can balance hemolysis sufficiently to prevent anemia at the expense of marked reticulocytosis. Stable hemolytic anemias can be dramatically affected by infections that induce production defects similar to that seen in anemia of chronic disease. Even minor infections have been reported to drop hemoglobin concentrations from 12 g/dl to nearly 7 in one week(103).

Causes of Folate Deficiency

Mechanism	Cause
Inadequate Intake	Alcoholism Nutritional Deficiencies
Increased Needs	Pregnancy Severe hemolysis Dialysis
Inadequate Absorption	Tropical sprue Celiac sprue Crohn disease Lymphoma Amyloid Diabetes S.B. Surgery
Drug interference with folate metabolism	DHFR inhibitors Decreased folate absorption Unknown mechanism

Figure 32-Causes of Folate Deficiency

Hemolytic anemias can be divided into extravascular and intravascular. Of these two, extravascular hemolytic anemias are by far the most common. Most of these conditions are associated with inheritied abnormalities of hemoglobin, the cytoskeleton, or constituents of the RBC membrane. Macrophages clear the defective RBCs and efficiently recycle iron for new hemoglobin synthesis. These hemolytic anemias tend to be fairly well tolerated because RBCs are cleared and recycled through stimulation of normal pathways.

Intravascular hemolysis is a completely different story. RBCs were never intended to lyse in the circulation and no efficient system exists for recycling the cytoplasmic constituents of lysed RBCs. As a result, they tend to be lost in the urine. Free hemoglobin can be cleared by the reticuloendothelial system after being complexed to haptoglobin. Circulating haptoglobin is easily exhausted leading to hemoglobinuria, iron uptake by renal epithelial cells, and subsequent hemosiderinuria. Severe anemia can result acutely from intravascular hemolysis. The abnormalities that cause hemolysis may be intrinsic to the RBC itself (intracorpuscular) or RBC may be attacked by external forces (extracorpuscular.) All of the hemolytic intracorpuscular defects are inherited except one, PNH.

VI. Treatment and Related Issues

There are no reliable, specific, and inexpensive treatments available for PNH and no treatments have been studied in a randomized double-blind fashion. While the best care is often defined as supportive, there are guiding principles and there is controversy about future directions. The anemia is usually treated with both iron and folate to control attendant deficiencies that are universal in PNH. Many patients require transfusions at various points in the course of the disease. Some patients benefit from chronic corticosteroid therapy to slow RBC clearance and others appear to benefit from androgenic steroid therapy to increase production of RBCs derived from normal stem cells. The use of erythropoietin has been suggested and used by some(104), but its use is definitely controversial. Aplastic anemia usually does not respond to erythropoietin and the diseased clone in PNH is already massively expanded. When the combination of hemolysis and aplasia is severe enough, transfusion may be required. Typing

must be meticulous because minor mismatches can provoke severe complement-mediated lysis of the PNH III cells.

Many patients with PNH have diagnosable aplastic anemia as well. Patients with aplastic anemia alone often respond to immunosuppressive therapy with drugs like cyclosporine A. This drug appears to be efficacious in some patients with aplastic anemia and PNH(105).

Some patients die of bleeding secondary to the thrombocytopenia(22). Patients with severe thrombocytopenia and bleeding complications should be given platelet transfusions.

Thrombosis is the most common cause of PNH related death(22). Acute thromboses can be treated with full dose heparin even though low dose heparin is said to activate complement(106). The risk of hemolysis due to heparin therapy is minor(106). McMullin recently reported two patients with PNH complicated by Budd-Chiari syndrome (hepatic vein thrombosis) who completely cleared their clots with tissue plasminogen activator and remained disease free for 2 and 6 years respectively(107). Thrombotic complications can usually be prevented with warfarin anticoagulation.

There is no therapy currently that prevents infection or acute leukemia. These patients may actually live for many years in spite of fairly severe abnormalities of circulating cells and the marrow. Furthermore, Hillmen report a 15% rate of spontaneous and permanent remission(22).

Recently, bone marrow transplantation is being used to treat difficult cases and is showing considerable promise(88, 89, 108) but is also controversial.

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