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The Antibody Repertoire of Patients with Paroxysmal Nocturnal Hemoglobinuria and Myelodysplastic Syndrome

Dissertation
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إهداء إلى خطيبتي الغالية مها
Für meine Verlobte Maha

Contents

I. Zusammenfassung

Der Name paroxysmale nächtliche Hämoglobinurie (PNH) steht für eine erworbene Erkrankung, bei der die Erythrozyten innerhalb der Blutgefäße lysieren (intravasale Hämolyse). Aufgrund einer erworbenen Veränderung in ihrem Genom weist ein Teil der Blutzellen bei Patienten mit PNH einen Defekt einer Gruppe von Oberflächenproteinen auf, die nicht konventionell, sondern über ein besonderes Ankermolekül (GPI-Anker) mit der Zellmembran verbunden sind. Zwei dieser Moleküle regulieren das Komplement und sind die einzigen Komplement-Regulatoren auf den Erythrozyten. Wenn die Komplementkaskade nicht an der Membran reguliert wird, kommt es zu einem erheblichen Zellschaden bis hin zur Lyse der Zelle. Das Auftreten der GPI-defizienten Zellen im Blut der PNH-Patienten beruht auf einer Vermehrung eines oder weniger einzelner Zellklone mit der oben beschriebenen Veränderung in ihrer Erbsubstanz im Knochenmark (klonale Expansion). Der Mechanismus der klonalen Expansion ist bis zum heutigen Tag nicht geklärt. Eine Hypothese postuliert einen Selektionsmechanismus, durch den die normalen Zellen erkannt und eliminiert werden. Es bleiben die GPI-defizienten Zellen übrig, und das Knochenmark und das periphere Blut werden von den GPI-defizienten Zellen dominiert. Die Identifizierung solcher autoreaktiven Antikörper erfolgte mittels SEREX (Serological analysis of antigens by recombinant expression cloning). Dazu wurde eine humane fötale Leber-cDNA-Expressionsbank mit 10 Seren von PNH-Patienten, 5 aplastischer Anämie, 10 myelodysplastischem Syndrom (MDS) und 20 gesunden Individuen auf immunreaktive Klone untersucht. Dabei konnten insgesamt zwei reaktive Klone identifiziert werden, welche in $CD34^+$ Zellen sowohl aus gesundem Knochenmark als auch aus PNH Knochenmark auf gleiche Weise exprimiert werden. Bei 3/10 PNH-Patienten wurde eine Antikörper-Reaktion gegen M-Phase Phosphoprotein 1 (MPHOSPH 1) und bei 4/10 PNH-Patienten eine Antikörper-Reaktion gegen Desmoplakin identifiziert. Weder mit Seren von Patienten mit aplastischer Anämie noch mit MDS Seren waren Reaktionen zu beobachten. Wir fanden auch eine Antikörper-Reaktion in einem der 20 gesunden Probanden gegen Desmoplakin, aber mit einem viel niedrigen Titer als bei PNH-Patienten (10^2 zu 10^5). Kein Unterschied konnte festgestellt werden bei den DNA-Sequenzen beider Klone aus PNH $CD34^+$ Zellen und denen aus gesunden $CD34^+$ Zellen. Aus den Ergebnissen lässt sich schließen, dass MPHOSPH1 und Desmoplakin zwei Autoantigene sind, die vom Immunsystem der PNH-Patienten erkannt werden. Diese Ergebnisse können zu einem besseren Verständnis der Pathophysiologie der Krankheit beitragen.

II. Summary

Paroxysmal Nocturnal Hemoglobinuria (PNH) is a consequence of a non-malignant clonal expansion of hematopoietic stem cells with a somatic mutation of the PIG-A gene. Mutations in the PIG-A gene lead to a deficiency of glycosyl phosphatidyl inositol–anchored proteins (GPI-APs). This deficiency leads to a complete absence of complement regulating proteins on the surface of red blood cells and thus explains the intravascular hemolysis of PNH. However, this mechanism does not account for the clonal expansion of GPI-AP-deficient cells. Since the PIG-A mutation does not confer to an intrinsic growth advantage within the bone marrow; this suggests a second event to be responsible for the clonal expansion of mutant cells. A hypothesis assumes a defect in the immune system leading to an immune attack directed against specific antigens, which have yet to be identified. In order to identify the auto-antigens in PNH patients, a human fetal liver cDNA library was screened for hematopoietic stem/progenitor cells antigens using the SEREX (Serological analysis of antigens by recombinant expression cloning) approach. For the screening 10 PNH sera, 5 aplastic Anemia, 10 Myelodysplastic syndrome (MDS) and 20 healthy volunteers sera were used. Two antigens were identified that are constitutively expressed in $CD34^+$ cells from both healthy and PNH bone marrow. Three of 10 PNH patients were identified to exhibit an antibody response against M-phase phosphoprotein 1 (MPHOSPH1) and four of 10 PNH patients were identified with an antibody response against desmoplakin. A weak serological response in one of 20 healthy volunteers against desmoplakin was detected, however at a much lower titer than in PNH patients (10^2 in normal serum VS 10^5 in PNH sera). No response to MPHOSPH1 or desmoplakin was detected in 5 patients with aplastic anemia or the 10 patients with MDS. Moreover, no difference between the cDNA sequences of the clones (MPHOSPH1 and desmoplakin) isolated from PNH $CD34^+$ cells and from healthy $CD34^+$ cells were identified.

From the results, it can be concluded that MPHOSPH1 and desmoplakin are two auto-antigens that are recognized by the immune system of PNH patients. The analysis of the mechanisms underlying this auto-immunity might contribute to a better understanding of the clonal expansion associated with this disease.

III. Glossary

AA	Aplastic anemia
APC	Antigen presenting cell
BCIP	5-bromo-4-chloro-3-indoly-phosphate
CDR	Commonly deleted region
cGMP	Cyclic guanine mono-phosphate
CMML	Chronic myelomonocytic leukemia
DAF	Decay accelerating factor
dNTPs	deoxynucleotides triphosphates
DSP	Desmoplakin
FAB	French-American-British
GPI	Glycosyl phosphatidyl inositol
HMGA2	High mobility group AT-hook protein 2
IPSS	International Prognostic Scoring System
IPTG	isopropyl-1-thio-β-D-galactopyranoside
IS	Immunosuppressive therapy
LGLL	large granular lymphocyte leukemia
MAC	Membrane attack complex
MDS	Myelodysplastic syndromes
MIRL	Membrane inhibitor of reactive lysis
MPHOSPH1 or MPP1	M- Phase phosphoprotein
MPS	Myeloproliferative disease
NBT	Nitroblue tetrazolium
NK	Natural Killer
MOPS	3-(N-Morpholino)-propanesulfonic acid

NOS	Nitric oxide synthesis
PIG-A	phosphatidylinositol glycan anchor biosynthesis, class A
PNH	Paroxysmal Nocturnal Hemoglobinuria
RA	Refractory anemia
RARS	Refractory anemia with ringed sideroblasts
RAEB	refractory anemia with excess blasts
RAEB-T	Refractory anemia with excess blasts in transformation
SEREX	Serological analysis of antigens by recombinant expression cloning
TCR	T cell receptor
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization
WT1	Wilms' tumor-1 protein

IV-Introduction

1. Paroxysmal Nocturnal Hemoglobinuria (PNH)

1.1. Definition

Paroxysmal Nocturnal Hemoglobinuria (PNH) is an acquired disorder of hematopoietic stem cells; its estimated prevalence is about $1/10^5$ people. Three recent large clinical studies on patients with PNH indicated that it may occur at any age from children as young as 8 months to adults up to 82 years of age and that it occurs most frequently in adults of age 30–50. The clinical manifestation of PNH is complex involving primarily three sets of symptoms: hemolysis with acute exacerbation (Figure 1), cytopenia of variable severity, and a tendency for abnormal thrombosis (Nishimura *et al*, 1999a).

Figure 1:Urine samples from a patient with PNH. Urine sampled at 7 am is dark, containing a large amount of free hemoglobin, which leaks out of PNH red cells as they burst. The urine generally clears during the day.

1.2. Etiology

1.2.1. Glycosyl phosphatidyl inositol-linked antigens and PNH

The fact that a variety of proteins attach to the cell membrane by glycolipid structure is well known since 1980s. These proteins are attached to the cell membrane with the same basic backbone consisting of a phosphatidyl inositol, a single glucosamine, three mannoses and an ethanolamine. The glycosyl phosphatidyl inositol (GPI) structure is depicted in (Figure 2).

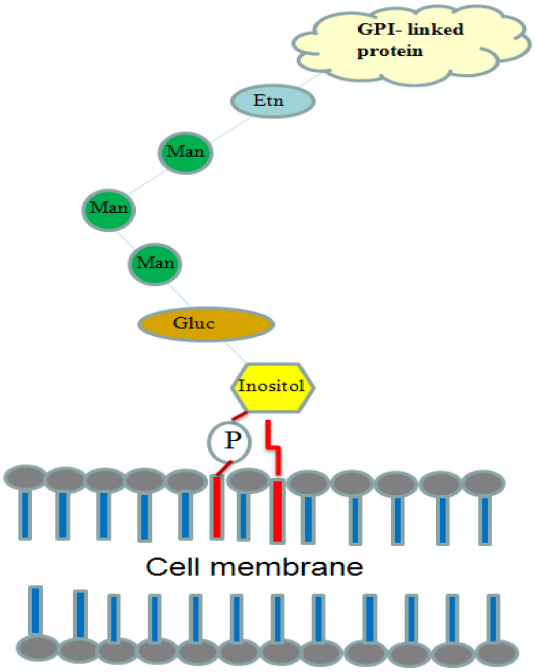


Figure 2: Structure of GPI anchor. GPI anchor consists of a phosphatidyl inositol, a single glucosamine, three mannoses and an ethanolamine—a Glycosyl phosphatidyl inositol.

1.2.1.1. GPI-anchor biosynthesis

PIG genes are involved in the biosynthesis of the GPI-anchor and its binding to proteins, which takes place in the endoplasmic reticulum ER (Figure 3). GPI-linked proteins can be released by specific phospholipases, for example GPI-PLS and GPI-PLD (Tashima *et al*, 2006). In trypanosomes, GPI-PLC-controlled release of the variant specific glycoprotein, the main coat protein, enables the parasite to evade the host immune response. However, the functional role of GPI-PLC and GPI-PLD in human tissues remains elusive. Good lateral mobility, which is another characteristic of GPI-anchor, is likely to be relevant for many GPI-linked proteins that require the clustering of GPI-anchor molecules in the association with microdomains, or “rafts”, enriched in glycophospholipids and cholesterol. The association with microdomains contributes to the specific surface distribution of GPI-linked proteins and aids in the recruitment of accessory molecules for the cell signaling (Horejsi *et al*, 1998).

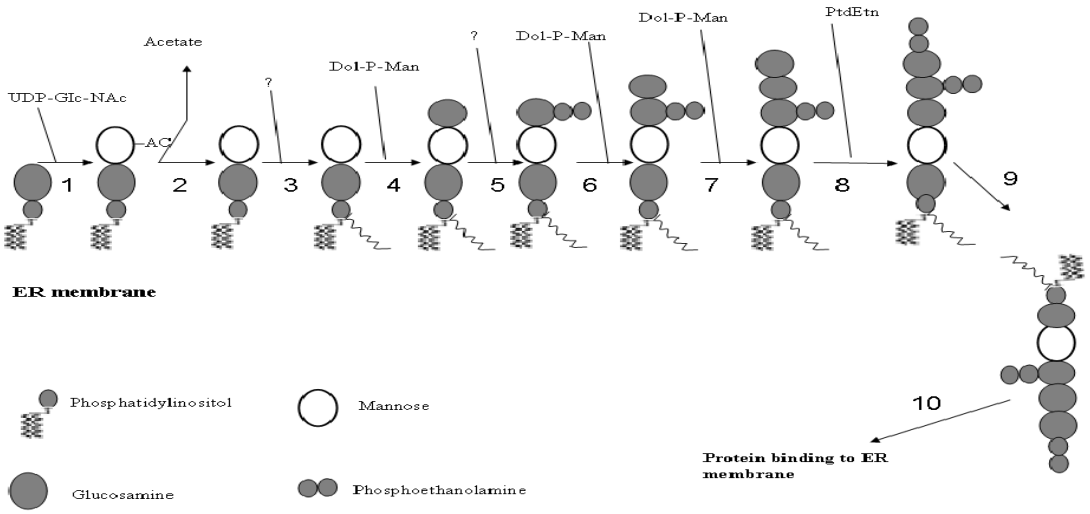


Figure 3: GPI-anchor biosynthesis. Steps (1-8) are the assembling of GPI-anchor precursors by adding sequential of components to phosphatidyl inositol. (Steps 9 and 10) GPI-anchor precursors linked to the carboxyl terminus of the protein to form GPI-anchored protein. (Modified according to Takeda and Kinoshita, 1995)

The PIG-A gene is located on the short (p) arm of the X chromosome at position 22.1, from base pair 15,247,500 to base pair 15,263,565 (Figure 4). The PIG-A gene is approximately 17 kilo bases (kb) long and has six exons. Exon1 is non-coding, and exon 2 accommodates almost half of the PIG-A gene-coding region (Iida *et al*, 1994b). Exon 5 contains sequences that have homology to the Glc-NAc-transferase of Salmonella typhimurium and glycosyltransferases in plants and thus might be the binding site of UDPGlcNAc (Bessler *et al*, 1994b). Exon 6 contains the trans-membrane domain of PIG-A. The PIG-A cDNA consists of 3589 base pairs (bp) with an open reading frame of 1452 bp encoding a putative protein of 484 amino acids. The PIG-A gene maps to the short arm of the X chromosome at X p22.1. A non-functional processed pseudo gene was mapped to 12 p21.

1.2.1.2. PIG-A gene mutations

A single mutation can cause the loss of GPI-linked proteins on both male and female somatic cells. Thus, in males the inactivation of one PIG-A allele is sufficient to cause the loss of GPI-linked proteins on the cell surface. In females, the inactivation of PIG-A allele when it is on the active X chromosome will lead to the loss of GPI-linked proteins on the cell surface (due to random X-inactivation this is expected to occur in about half of the cells). Therefore, in both male and female cells, a single mutation (1 hit) can be sufficient to inactivate PIG-A protein function resulting in the loss of GPI linked proteins on the cell surface. All other genes involved in the GPI-anchor biosynthesis are autosomal. Therefore, the loss of GPI-anchored

proteins would require two separate mutations on the two different alleles, an event that is extremely unlikely to occur.

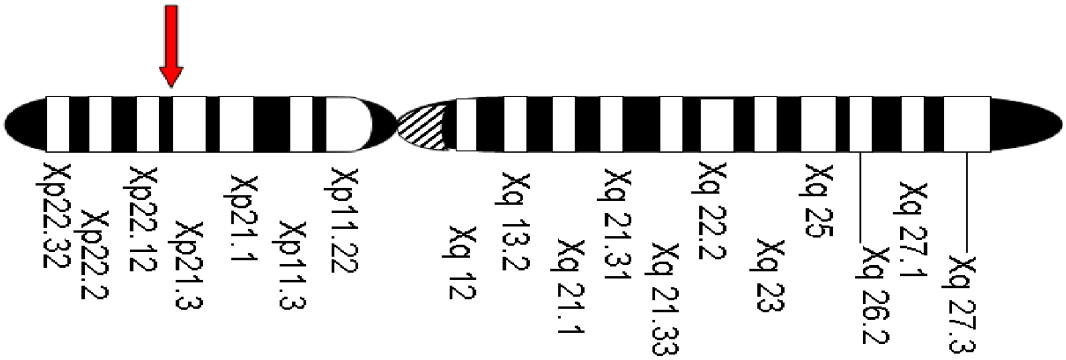


Figure 4: Location of PIG-A gene on chromosome X. The PIGA gene is located on the short arm of the X chromosome at the position 22.1 from base pair 15,337,577 to base pair 15,353,659 of the chromosome.

To date, more than 180 mutations are known to be found in GPI-deficient blood cells from patients with PNH (Bessler & Hiken, 2008). The majority of PIG-A gene mutations are frame shift mutations that predict an inactive PIG-A protein and a loss of glycosyltransferase activity. The mutations are distributed over the entire coding region with no obvious clustering. Only 16 out of 174 mutations were reported to be present in more than one PNH patient (Nishimura *et al*, 1999b). There is some clustering of missense mutations over the coding region of exon 2. Some of these missense mutations were shown to cause only a partial deficiency in GPI-linked proteins on the cell surface. This indicates that the mutant glycosyltransferase has some residual activity (Bessler *et al*, 1994c).

1.2.2. Intravascular Hemolysis in PNH

The work of Ham (Ham *et al*, 1948) in the 1930s first revealed that the hemolysis in PNH was due to the effect of a serum factor on abnormal PNH red cells. Rosse et al (Rosse & Ware, 1995) proceeded to show that this factor was the complement, that when activated led to the intravascular hemolysis of PNH red cells. The characteristic symptoms of PNH can be attributed to the intense intravascular hemolysis and the resulting free plasma hemoglobin. This appears to be due to the absorption of nitric oxide by free hemoglobin and since nitric oxide is critical for smooth muscle function. During intravascular hemolysis, hemoglobin is released into the plasma where it is normally cleared by the hemoglobin's scavengers:

haptoglobin, CD163, and hemopexin. Haptoglobin-hemoglobin complexes bind to CD163 on the surface of macrophages/monocytes initiating endocytosis and degradation of the complex. Hemoglobin also releases ferric heme on oxidation, which is bound by hemopexin and degraded by hepatocytes in the liver (Rother *et al*, 2005). Excessive hemolysis saturates and depletes these hemoglobin removal systems and leads to a buildup of hemoglobin and heme in the plasma. Plasma hemoglobin and heme mediate direct pro-inflammatory, proliferative, and pro-oxidant effects on vessel endothelial cells. NO is normally generated from L-arginine in vessel endothelial cells by the enzyme nitric oxide synthesis (NOS). NO maintains smooth muscle relaxation and inhibits platelet activation and aggregation, thereby regulating vessel tone and promoting organ system homeostasis. During intravascular hemolysis, NO availability can be severely limited by its reaction with oxy-hemoglobin (NO scavenging) and by the breakdown of the substrate for NO synthesis, L-arginine, by the red cell enzyme arginase, despite elevated levels of NOS (decreased NO synthesis). NO depletion results in decreased activation of guanylate cyclase, an enzyme required for the generation of cyclic guanine mono-phosphate (cGMP). Decreased cGMP levels disrupt regulation of smooth muscle tone resulting in dystonias, including systemic and pulmonary hypertension, erectile dysfunction, dysphagia, and abdominal pain. Decreased cGMP levels through the depletion of NO can also lead to platelet activation and aggregation, promoting clot formation (Figure 5).

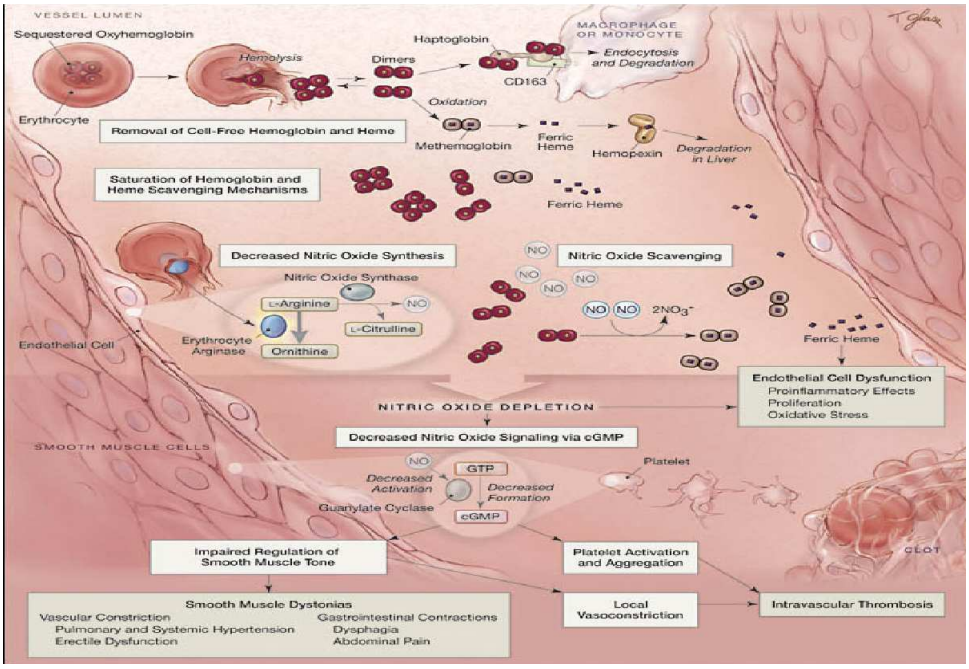


Figure 5: Pathobiological effects of cell-free plasma hemoglobin and nitric oxide (NO) depletion during intravascular hemolysis. (Rother *et al*, 2005).

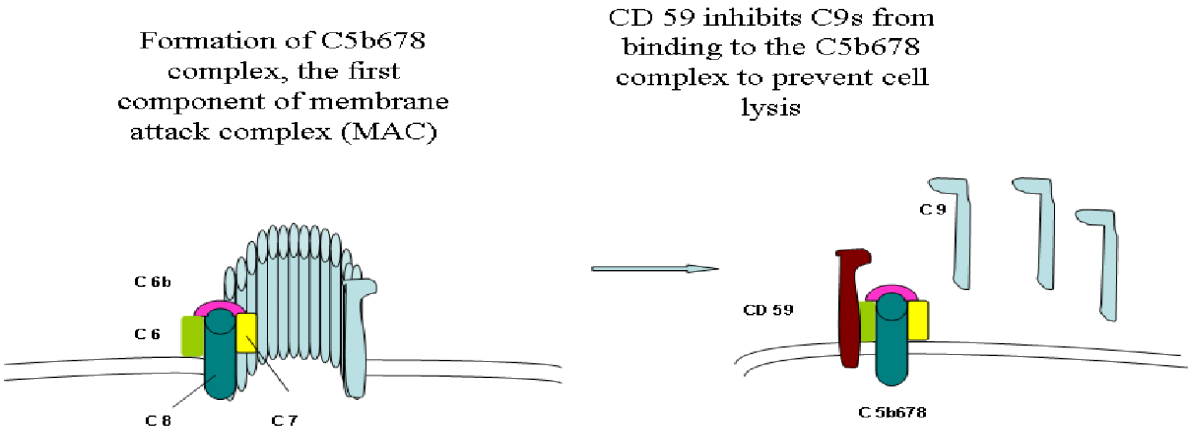


Figure 6: Regulation of complement activity. Binding of poly C9 to the C5b678 complex leads to the formation of membrane attack complex (MAC), the binding could be inhibited by CD59. (Modified, from Department of Biology, Davidson College, 2005)

The functions of the GPI-linked antigens are divergent. At least two are important in the control of complement: Decay accelerating factor (DAF or CD55), which controls the early part of the complement cascade by regulating the activity of the C3 and C5 convertases. Thus, CD55 deficiency initially appeared to explain the sensitivity of PNH red cells to complement. However, the observation that individuals with inherited CD55 deficiency (Inab-phenotype) did not suffer from hemolysis proved that deficiency of CD55 does not cause the hemolysis in PNH. The second protein is Membrane inhibitor of reactive lysis (MIRL or CD59) which is GPI-linked protein and it was identified in 1989. CD59 inhibits terminal complement by preventing the incorporation of C9 onto C5b-8 (Figure 6 and 7) and therefore preventing the formation of the membrane attack complex (MAC). In 1990, an individual with inherited isolated deficiency of CD59 was described with many features similar to classic PNH, such as intravascular hemolysis with hemoglobinuria and thrombosis of the cerebral veins (Iida *et al*, 1994a). Therefore, CD59 deficiency is the abnormality that is responsible for the hemolysis and thrombosis typical of PNH.

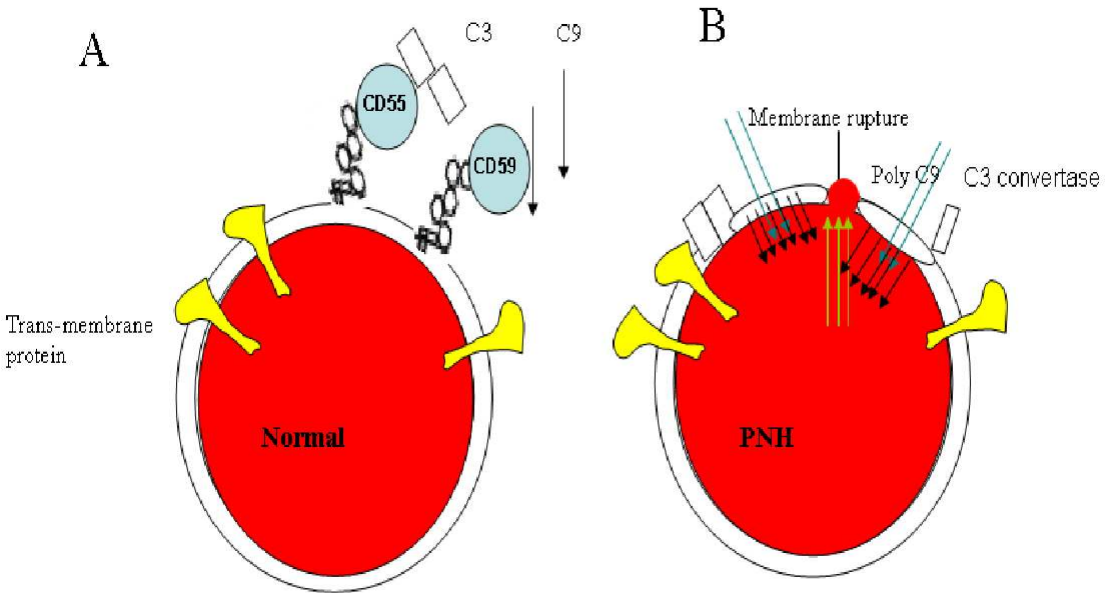


Figure 7: Complement mediated lysis of GPI-anchor deficient erythrocytes in PNH. (A) by normal blood cells: CD55 inhibits the formation or destabilizes the C3 confertase and CD59 protects the membrane from attack by the C5-C9 complex. (B) In PNH: the membrane attack complex can be formed because of the deficiency of both CD55 and CD59; the complex ruptures the membrane and the cell lysis. (Modified according to Lyakisheva and Schubert 2002).

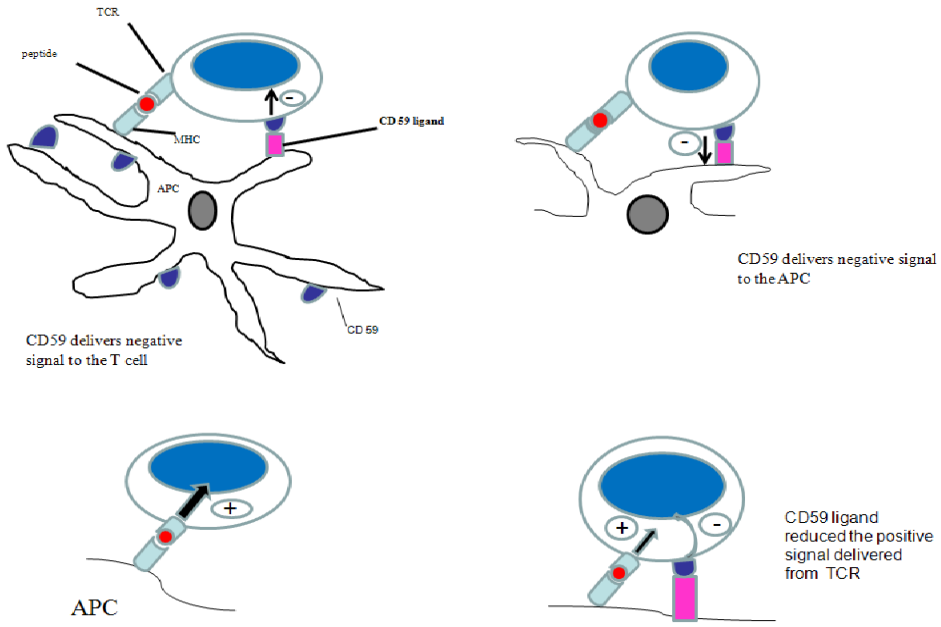


Figure 8: Effect of CD59 on T cell-APC interactions. The interaction of T cells with a specific receptor of an APC results in an inhibitory signal between the two cells, which reduces the strength of positive signals delivered through the TCR. (Modified, from the Department of Biology, Davidson College, 2005)

CD59 influences the outcome of a T cell response to a given antigen. Longhi et al (Longhi *et al*, 2006) have demonstrated in 2006 that direct interaction between CD59 on a T cell and a specific receptor on an antigen presenting cell (APC) results in an inhibitory signal being transmitted to both the T cell and the APC. Due to this inhibitory signal, down-modulation of APC activity and consequently T cell activity results as well. They also postulated that CD59 on T cells may reduce the strength of the positive signal transduction pathway delivered through the T cell receptor (TCR). A diagram for CD59 interacting with T cells and APCs is shown in (Figure 8).

1.2.3 Relative growth advantage of the PNH clone

PNH is a disorder in which an abnormal clone or small number of clones expand to replace almost the entire hematopoietic stem cell pool, but these clones do not have any "malignant" tendency in that they appear to be regulated in a normal manner with no tendency to metastasize beyond the normal hematopoietic compartment. Although GPI-deficient cells with PIG-A mutations occur very frequently at low levels in normal individuals they do not expand in competition with the normal hematopoietic cells. Hillmen and Dacie (Hillmen *et al*, 1995) first proposed that in order to develop PNH two things are required: first, the occurrence of a GPI-deficient clone arising in a multipotent hematopoietic stem cell; second, a second event that favors the expansion of the PNH clone over the residual normal hematopoiesis—a relative growth advantage for the PNH cells. The clue to this second event is the close relationship between aplastic anemia and PNH. It appears that normal hematopoiesis is suppressed by the immune system, presumably either directly or indirectly through one or more GPI-linked antigens, and therefore this attack spares the GPI-deficient PNH clone. Thus, in an environment where there is intense pressure for hematopoiesis (for example in aplastic anemia), the PNH clone is driven to produce mature hematopoietic cells and expands to fill the void left by the aplastic process. The exact mechanism for the relative growth advantage of PNH cells remains unclear. In 2006, Inoue et al (Inoue *et al*, 2006a) reported 2 patients with PNH whose *PIGA*-mutant cells (and not normal cells) had an acquired rearrangement of chromosome 12. In both cases, the chromosome had a break within the 3' untranslated region of *HMGA2*, the architectural transcription factor gene deregulated in many benign mesenchymal tumors, which caused ectopic expression of *HMGA2* in the bone marrow. These observations suggest that aberrant *HMGA2* expression, in concert with mutant *PIGA*, accounts for clonal hematopoiesis in both patients and suggest the concept of PNH as a benign tumor of the bone marrow.

1.2.4. Clonal selectivity in PNH

The study of Horikawa et al (Horikawa *et al*, 1997) confirmed that the blood cells obtained from patients with PNH are less susceptible to both spontaneous and ligand induced apoptosis in vitro than those from healthy volunteers. The resistance to apoptosis was evident in the granulocytes but unclear in the lymphocytes.

The PIG-A gene is responsible for the membrane defect of PNH cells and it might play an important role in the resistance to apoptosis, although the study of Horikawa et al showed no correlation between resistance to apoptosis and the proportion of cells with PNH phenotype.

In 2002 Nagakura et al (Nagakura *et al*, 2002a) found that leukemic cells with PIG-A mutations (GPI $^{-}$ cells) are less susceptible than the control counterparts (GPI $^{+}$cells) to kill by natural Killer (NK) cells in vitro, which supports a selective survival advantage theory. They also suggested that NK cells might spare PIG-A mutant cells from killing in vivo as well as in vitro. The study showed also that neither CD55 nor CD59 were the target antigens for the NK.

2. Myelodysplastic syndromes (MDS)

2.1. Definition

Myelodysplastic syndromes (MDS) are a group of acquired neoplastic disorders of multipotent hematopoietic stem cells characterized by increasing bone marrow failure with quantitative and qualitative abnormalities of all three cell lines. A hallmark of the disease is an active but ineffective hematopoiesis leading to pancytopenia. MDS were recognized for more than 50 years and were called preleukemia, smoldering leukemia, oligoblastic leukemia, and refractory anemia. There is a tendency to progress to AML, although death can also occur before this develops (Heaney & Golde, 1999). The term MDS reflects the presence of dysplasia in bone marrow and peripheral blood. Dysplasia may reflect disordered maturation and fragmentation of the nuclear structures, both of which are signs of increased apoptosis (Kouides & Bennett, 1997).

MDS were probably first described in 1900 by Leube as "leukanaemie", that at the time were thought to have an infectious etiology. After that, all patients who developed acute leukemia after having macrocytic anemia were given a diagnosis of “pre-leukemia” until the 1970s, when it was realized that many such patients never developed acute leukemia, but instead died

of complications from the cytopenias. The “pre-leukemia” terminology faded away, and the term “myelodysplastic syndrome” became widely accepted. Signs and symptoms of anemia, accompanied by infectious or bleeding complications, predominate in MDS, with some patients having systemic symptoms or features of autoimmunity, perhaps indicative of the pathogenesis of their disease (Nimer, 2008).

2.2. Classification of MDS

The first French-American-British (FAB) Cooperative Group meeting identified two broad categories of “dysmyelopoietic syndrome”. In 1982, they expanded their classification to the modern five subcategories of MDS: Refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEB-T) and chronic myelomonocytic leukemia (CMML) (Bennett *et al*, 1982). Despite its obvious limitations, the FAB classification has remained the standard for more than two decades. Nonetheless, it was substituted by the World Health Organization (WHO) classification. The WHO classification incorporates many of the concepts and definitions of the FAB system, but it also recognizes recently published data to refine the definition of some subtypes and thus to improve their clinical relevance (Vardiman *et al*, 2002b). The most important difference between the WHO and FAB classifications is the lowering of the blast threshold for the diagnosis of AML from 30% to 20% blasts in the blood or bone marrow. As a result, the FAB category RAEBT is eliminated from the WHO classification (Greenberg *et al*, 1997c). Most recently, the WHO has evolved a new classification scheme (2008) which is based more on genetic findings (Table 1). However, morphology of the cells in the peripheral blood, bone marrow aspirate, and bone marrow biopsy is still the screening test used in order to decide which classification is best and which cytogenetic aberrations may be related.

Other changes include a refinement of the definitions for the lower-grade lesions, RA, and RARS, and the addition of a new category, refractory cytopenia with multi-lineage dysplasia (RCMD). Two subtypes of RAEB, RAEB-1 with 5% to 9% marrow blasts and RAEB-2 with 10% to 19% marrow blasts, are also recognized. They take into account data published by the International MDS Risk Analysis Workshop that patients with 10% or more blasts in the bone marrow have a worse clinical outcome than do those with fewer blasts. The WHO classification also recognizes the “5q− syndrome” as a unique, narrowly defined entity. Lastly, because of the controversy as to whether chronic myelomonocytic leukemia (CMML)

is a myelodysplastic or a myeloproliferative disease, this disorder was placed in a newly created disease group, MDS/MPD. The International Prognostic Scoring System (IPSS) relies on the primacy of the number of cytopenias, cytogenetic profile, and the percentage blasts in the bone marrow into one of four prognostic categories: low risk, intermediate 1 risk, intermediate 2 risk, and high risk (Table 2).

Table 1. WHO classification of MDS (new and old system).

WHO classification old system	WHO classification new system
Refractory anemia (RA)	Refractory cytopenia with unilineage dysplasia (Refractory anemia, Refractory neutropenia, and Refractory thrombocytopenia)
Refractory anemia with ringed sideroblasts (RARS)	Refractory anemia with ring sideroblasts (RARS) Refractory anemia with ring sideroblasts - thrombocytosis (RARS-t) (provisional entity) which is in essence a myelodysplastic/myeloproliferative disorder and usually has a JAK2 mutation (janus kinase) - New WHO classification 2008
	Refractory cytopenia with multilineage dysplasia (RCMD) includes the subset Refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS). RCMD includes patients with pathological changes not restricted to red cells (i.e., prominent white cell precursor and platelet precursor (megakaryocyte) dysplasia.
Refractory anemia with excess blasts (RAEB)	Refractory anemia with excess blasts I and II. RAEB was divided into *RAEB-I (5-9% blasts) and RAEB-II (10-19%) blasts, which has a poorer prognosis than RAEB-I. Auer rods may be seen in RAEB-II which may be difficult to distinguish from acute myeloid leukemia.
Refractory anemia with excess blasts in transformation (RAEB-T)	The category of RAEB-T was eliminated; such patients are now considered to have acute leukemia. 5q- syndrome, typically seen in older women with normal or high platelet counts and isolated deletions of the long arm of chromosome 5 in bone marrow cells, was added to the classification.
Chronic myelomonocytic leukemia (CMML)	CMML was removed from the myelodysplastic syndromes and put in a new category of myelodysplastic-myeloproliferative overlap syndromes.
	5q- syndrome
	Myelodysplasia unclassifiable (seen in those cases of megakaryocyte dysplasia with fibrosis and others)
	Refractory cytopenia of childhood (dysplasia in childhood) - New WHO classification 2008

Table 2. International Prognostic Scoring System IPSS (Greenberg *et al*, 1997a)

Risk group	Total score	Median survival, y	Time for 25% to progress to AML, y
Low	0	5.7	9.4
Intermediate-1	0.5-1.0	3.5	3.3
Intermediate-2	1.5-2.0	1.2	1.1
High	≥ 2.5	0.4	0.2

2.3. Causes & Risk Factors

Most cases of MDS have no known cause, but some factors were determined to increase the risk. Advancing age is perhaps the most common risk factor, since it rarely occurs in people under the age of 60. Other risk factors include smoking, long-term exposure to benzene and prior treatment with chemotherapy or radiation (West *et al*, 1995).

2.4. Pathophysiology of MDS

Both MDS and myeloproliferative diseases can be thought of as preleukemic disorders. AML is characterized by a block in differentiation, but also the ongoing ability of the myeloblasts (and leukemic stem cells) to survive and proliferate. In contrast, MDSs have impaired differentiation, making it likely that a second mutation, which allows the blasts to survive and proliferate, is needed for the disease to progress to AML. Likewise, in the myeloproliferative diseases, where proliferation is enhanced and differentiation is initially normal, the disease most likely progresses to acute leukemia when a second hit impairs differentiation.

Increased macrophage function with increased cytokine secretion, changes in microvessel density, immunologic abnormalities (which are often found in aplastic anemia patients as well) and absence of circulating NK T cells wereidentified in patients with MDS (Fujii *et al*, 2003).

2.4.1. Cytogenetic abnormalities

The search for the mechanistic basis of MDS was fueled largely by the identification of recurrent cytogenetic abnormalities that are associated with specific clinical scenarios. The common chromosomal abnormalities found in MDS include abnormalities in 17p, loss of Y, 5q–, 7q– or monosomy 7, trisomy 8, 11q23 abnormalities, del 12p and 20q–, and in half of the patients, a normal chromosome pattern. None of these abnormalities is specifically associated

with MDS, as all can be seen in AML and some in the myeloproliferative diseases. The cause of MDS is usually unknown, although it can occur after exposure to radiation, certain environmental toxins, such as benzene, or after treatment of a primary malignancy with alkylating agents or topoisomerase II inhibitors (PedersenBjergaard *et al*, 1995).

The presence of t (15; 17), t (8; 21), inv (16), and other specific abnormalities in AML but not MDS seems to reflect true differences in their biology. Recurrent chromosomal translocations in AML produce fusion transcription factor proteins that act as potent transcriptional repressors (most likely targeting genes that are required for normal hematopoietic cell differentiation), whereas some fusion proteins, such as those that contain the transcriptional regulator MLL, appear to function as potent activators of (Hox) gene expression. Deletions, numerical abnormalities, and unbalanced translocations are more commonly seen in MDS, and translocations specifically associated with MDS are rare.

The t (3; 21), which is documented in MDS and CML blast crisis, is one of the first recurrent MDS-associated cytogenetic abnormalities to be molecularly deciphered. The translocation rearranges the AML1 and MDS1–EVI-1 genes, fusing the N terminus of AML1 with a small portion of MDS1 and nearly all of EVI-1. Both components of the AML1–MDS1/EVI-1 fusion protein appear to play critical roles in deregulating hematopoiesis. AML1 (also known as CBFα or Runx1, for Runt-related protein 1) binds DNA with its non-DNA binding partner CBFβ. Lack of either AML1 or CBFβ is embryonically lethal because of the absence of definitive hematopoiesis and a distinct pattern of central nervous system hemorrhage (Wang *et al*, 1996;Okuda *et al*, 1996).

The AML1 gene is mutated at low frequency in MDS, but at much higher frequency in AML M0, and in treatment-related or radiation-induced MDS (Harada *et al*, 2003). Whereas the mutations found in M0 AML are often bi-allelic, those found in MDS are generally mono-allelic and result in AML1 insufficiency rather than generating a dominant negative protein. Most of the AML1 mutations in AML cluster in the Runt (DNA binding) domain and impair the binding of AML1 to DNA but not to CBFβ. The AML1 mutations, found in MDS, occur more often in the C-terminus of the protein and truncate the protein, eliminating its transactivating domain (Osato, 2004).

Some MDS patients with an interstitial deletion within the long arm of chromosome 5 (5q–) have the "5q– syndrome," whereas others do not. The 5q– syndrome, as defined by the WHO,

consists of an isolated 5q– cytogenetic abnormality, associated with macrocytic anemia, a normal or elevated platelet count, unilobular megakaryocytes, and a low propensity to develop AML (Vardiman *et al*, 2002a).

Although the presence of 20q– in MDS has a favorable prognosis, the critical genes deleted from this region also remain unknown. A gene that encodes a histone-binding protein within the commonly deleted region (CDR) on chromosome 20q12 was identified. The CDR contains a polycomb group gene, the L (3) MBTL1 gene (MacGrogan *et al*, 2001).

Several laboratories have focused on the commonly deleted region on 7q that is associated with MDS. Given the involvement of polycomb and trithorax group genes in AML and MDS and the importance of Hox gene expression in hematopoiesis and leukemogenesis, members of this family continue to be interrogated, such as the MLL5 gene (a member of the MLL family of genes), which is deleted by the 7q– deletion (Side *et al*, 2004).

Several publications describe the transcript profiles of purified stem cell fractions obtained from the bone marrow of patients with MDS (isolating either $CD34^{+}$ cells or AC133 expressing cells). Among the over expressed genes, the Delta-like protein (Dlk1) reported to be up-regulated by several groups (Miyazato *et al*, 2001;Pellagatti *et al*, 2006). Consistent up-regulation of interferon-γ–inducible genes has also been reported: IFITM1 and IFIT1 mRNA levels, and TRAIL, another interferon stimulated genes are up regulated in a substantial fraction of the MDS patients.

2.4.2. Immune system and MDS

T-cell dysregulation is definitely associated with bone marrow failure in AA and large granular lymphocyte leukemia (LGLL), which share several clinical features with MDS. Immunosuppressive therapy (IST) in MDS was applied in cases with bone marrow hypocellularity; a clinical feature that is commonly associated with AA and observed in approximately 15% of MDS cases (Young & Maciejewski, 1997). Numerous abnormalities in function appear to contribute to impaired hematopoiesis including elevated plasma levels of several cytokines such as tumor necrosis factor-alpha (TNF-α) (Molnar *et al*, 2000) and interferon-gamma (IFN-γ) (Selleri *et al*, 2002), which are well-known contributors to AA pathogenesis. Many investigators have now confirmed that cytopenias are corrected by T-cell depleting immunotherapy in some MDS patient (Kochenderfer *et al*, 2002b). A hallmark of T-cell dominant autoimmunity is the expansion of T-cell clones with restricted diversity that

possess a limited T-cell receptor (TCR)-Vβ repertoire. Limitation in TCR-Vβ complementary-determining region 3 (CDR3) diversity and clonal expansion of T cells in association with bone marrow suppression suggests that escape from peripheral tolerance and recognition of self-antigens may contribute to suppressive hematopoiesis (Epling-Burnette et al, 2007). The identity of one antigen, that drives abnormal T-cell clonal expansion that contributes to impaired hematopoiesis in patients with a trisomy 8 cytogenetic abnormality, was confirmed (Sloand & Rezvani, 2008). Clonally expanded $CD8^+$ cells with reduced TCR-Vβ repertoire diversity and direct cytotoxicity of autologous trisomy 8 hematopoietic progenitors was observed in vitro, but no $CD8^+$ T-cell self-reactivity was observed in MDS patients with 5q- and monosomy7 chromosomal abnormalities (Sloand *et al*, 2005). The fact that a small number of trisomy 8 cells were present in bone marrow and could be detected using fluorescence *in situ* hybridization (FISH) long before cytogenetic conversion by standard metaphase karyotyping, suggests that trisomy 8 aneuploidy may be an early event in MDS. It was hypothesized that the CTL response was initially triggered by abnormal stem cells but the bone marrow failure was subsequently mediated by a 'bystander' CTL response against healthy hematopoietic cells. The Wilms' tumor-1 (WT1) protein, which is localized on chromosome 8, and thus over-expressed in MDS patients with trisomy 8, represents a well-defined target of self-reactive CTLs in immunosuppressive therapy (IST) responsive patients (Chen *et al*, 2004). Since the mechanisms contributing to immune pathophysiology and patient selection criteria for IST remain elusive in patients without trisomy 8, a search for predictive biomarkers is critically necessary. Expansion of PNH-type cells were reported in about 20% of the patients with lower-risk MDS and that phenomenon was linked in some studies to IST responsiveness (Wang *et al*, 2002). It was documented that inversion of the CD4/CD8 ratio was strongly associated with response to therapy. The loss of $CD4^+$cells was inversely correlated to the proliferative T-cell index before treatment in IST-responsive patients suggesting that proliferation and accelerated $CD4^+$ T-cell turnover may be important in disease pathogenesis (Zou *et al*, 2009). A discovery of T-cell receptor (TCR) antigen reactivity in patients with aggressive homeostatic proliferation, without trisomy 8, is necessary to determine the precise relationship between $CD4^+$ T cells, HLA DR15, and homeostatic turnover as an alternative mechanism of autoimmunity in MDS.

2.5. Clinical features of MDS

The evolution of MDS is often slow and the disease may be found by chance when a patient has a blood count for some unrelated reason. The symptoms of myelodysplastic syndrome are

those of anemia, infections or of easy bruising or bleeding. In some patients, transfusion dependent anemia dominates the course, while in others recurring infections or spontaneous bruising and bleeding is the major clinical problem. Different people are affected in different ways by myelodysplastic syndrome, and its symptoms can range from mild to very severe. Some people may only have long-term (chronic) anemia. Shortages of one or more types of blood cells cause most symptoms of MDS. Patients may have symptoms such as weight loss, fever and loss of appetite.

2.6. Laboratory findings

Peripheral blood pancytopenia is a frequent finding. The red cells are usually macrocytic or dimorphic but occasionally hypochromic; normoblasts may be present. The reticulocyte count is low. The cellularity of bone marrow is usually increased (Table 3). The typical morphological abnormalities in MDS include (Figure 13): megaloblasts, dissociated maturation of the nucleus and cytoplasm, abnormal multinucleated erythroblasts with three or more nuclei, and ringed sideroblasts in the erythrocytic lineage. In addition to hyper-segmented or hypo-segmented neutrophils (pseudo Pelger-Huët nuclear anomaly), reduced or missing granules, and peroxidase-negative neutrophils in the granulocytic lineage; and micromegakaryocytes, megakaryocytes with multiple, isolated disc-shaped nuclei and giant platelets in the megakaryocytic lineage.

Table 3: Diagnostic standards for Myelodysplastic syndrome.

1. Peripheral blood cytopenia.
2. Cellular bone marrow.
3. Chronic and refractory course.
4. Exclusion of underlying illnesses or administration of drugs.
5. Exclusion of other known blood disorders.

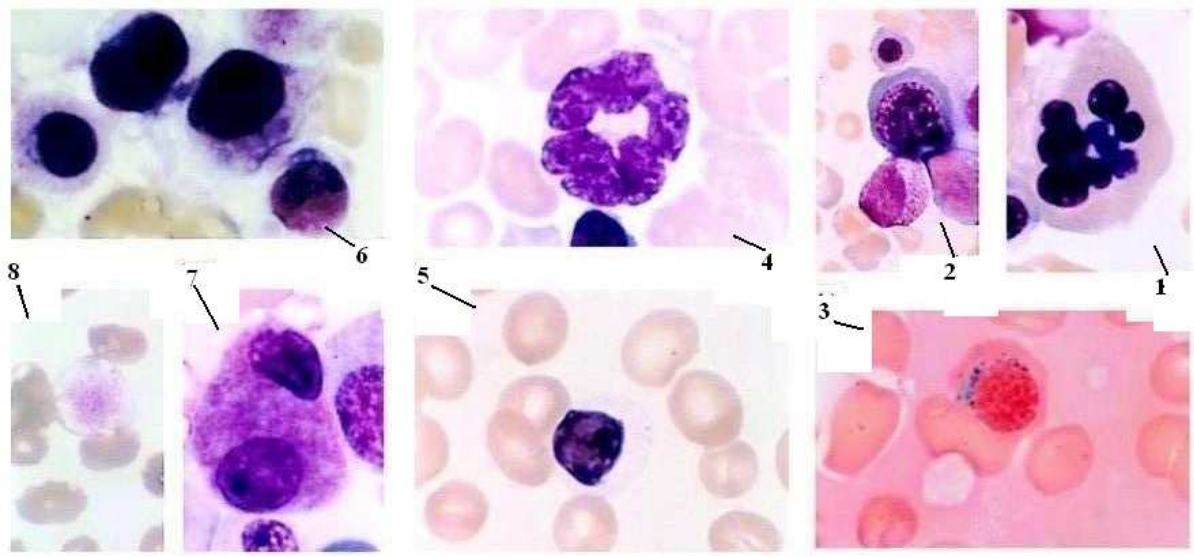


Figure 13: Morphological abnormalities in MDS. (1) Giant multinucleated erythroblast, (2) Megaloblast showing dissociated maturation of nucleus and cytoplasm, (3) Ringed Sideroblast, (4) Large hypersegmented neutrophil, (5) Mature neutrophil without nuclear lobes, (6) Micromegakaryocyte, (7) Micromegakaryocyte with disc-shaped, separated nuclei, (8) Giant platelets.

2.7. Treatment of MDS

The standard care for patients with myelodysplastic syndrome (MDS) and decreased blood counts is constantly changing. Supportive therapy, including transfusions of the cells that are missing (i.e. RBCs, platelets), and treatment of infections are the main treatments. The US Food and Drug Administration recently approved new drugs such as 5-azacytidine, 5-aza-2-deoxycytidine (Fenaux *et al*, 2007), and lenalidomide (List *et al*, 2005) for the treatment of myelodysplastic syndrome (MDS). Higher risk MDS patients usually need aggressive therapy, but much depends on the age and condition of the patient. Younger patients with high-risk disease are considered for front-line chemotherapy approaches followed by immediate allogeneic stem cell transplantation. For older patients, who constitute the majority, intensive chemotherapy is rarely considered. Instead, the strategy focuses on development of active and safe treatments for newly diagnosed patients as well as those who have failed the standard of care based therapies (Malcovati *et al*, 2007).

3. Aplastic Anemia

3.1 Definition

Aplastic anemia is defined as the failure of bone marrow to produce blood cell components. The hallmarks of the disease are pancytopenia and a hypo-cellular bone marrow.

3.2. Etiology

Most patients had a diagnosis of idiopathic disease in which no cause is apparent (Doney *et al*, 1997). Inherited forms of the disorder are rare and consist of Fanconi's anemia, dyskeratosis congenital, and Schwachman syndrome.

3.3. Clinical Presentation

The signs and symptoms of patients presenting with aplastic anemia are typically related to the decrease or absence of peripheral blood cellular components. The clinical presentation ranges from insidious to dramatic. Because platelets are depleted early in the process of the disease, dependent petechiae, bruising, gum bleeding, buccal hemorrhage, epistaxis, or retinal hemorrhage may be among the first presentations. Because of anemia, patients may complain of shortness of breath, fatigue, or chest pain. Neutropenia or leucopenia may result in fever, chills, or infections. Hepatosplenomegaly, lymphadenopathy, or bone pain are less common in patients with aplastic anemia, but these findings should alert the physician to other diagnoses, such as infection, leukemia, or lymphoma.

3.4. Pathophysiology of aplastic anemia

In most cases, aplastic anemia behaves as an immune-mediated disease. Cellular and molecular pathways were mapped in some detail for both effectors (T lymphocyte) and target (hematopoietic stem and progenitor) cells. The combination of exposure to specific environmental precipitants, diverse host genetic risk factors, and individual differences in the characteristics of the immune response likely account for the disease's infrequency, variations in its clinical manifestations, and patterns of responsiveness to treatment (Young, 2006).

Three decades ago, an immune mechanism was first implicated in the pathogenesis of aplastic anemia (Hirano *et al*, 2003b). Since then, accumulating evidence supports the hypothesis that immune mechanisms contribute to the pathogenesis of AA. Immunosuppressive therapies incorporating antithymocyte globulin, corticosteroids, cyclosporine, and/or cyclophosphamide

were successfully used in the treatment of patients with AA with response rates ranging from 50% to 80% (Bacigalupo *et al*, 1995), suggesting that pancytopenia and bone marrow failure in at least some AA patients are immunologically mediated. Furthermore, in vitro studies have also supplied supportive evidence for an immune-mediated suppression of hematopoiesis in AA. These include inhibitory effects of AA patient lymphocytes on hematopoietic stem/progenitor cell (HSPC) growth (Kagan *et al*, 1976), overproduction of myelosuppressive cytokines such as interferon-γ (IFN-gamma) and tumor necrosis factor-α (TNF-alpha) by patient bone marrow cells (Zoumbos *et al*, 1985), and an increased population of activated suppressor T cells. Taken together, these data suggest that at least some cases of AA involve autoimmune phenomena that target hematopoietic tissue, probably HSPCs. By establishing T-cell lines or clones from an involved organ and analyzing their specificity, investigators have successfully identified unknown target antigens in organ-specific autoimmune diseases and neoplasms (Steinman, 1996). Previous studies with T cells from AA patients have identified a pathogenic role for both $CD8^+$ and $CD4^+$ T cells. Peripheral blood T cells capable of suppressing in vitro growth of HSPCs belong mainly to the $CD8^+$ fraction (Harada *et al*, 1985). The importance of $CD4^+$ cytotoxic T cells in the pathogenesis of AA was also described. Several $CD4^+$ T-cell clones that can lyse autologous hematopoietic cells in an HLA class II-restricted fashion were isolated from AA patients (Nakao *et al*, 1997). The work of Hirano et al (Hirano *et al*, 2003a) proved that humoral immune responses to hematopoietic antigens could be detect in AA patients. Moreover, in 39 % of aplastic anemia patients included in the study antibodies against “kinectin” were documented.

4. PNH, MDS and aplastic anemia

PNH is closely related to aplastic anemia and certain forms of MDS. Many aplastic anemia patients have different degrees of PNH involvement. PNH may develop from aplastic anemia, or aplastic anemia and low counts might be a complication of PNH. Many specialists believe that aplastic anemia sets the conditions, which are advantageous for the growth of PNH stem cells and for the development of PNH (Nissen & Schubert, 2002). The clarification, as to why PNH and aplastic anemia occur so frequently together, might help us to understand the pathogenesis of these diseases and develop new specific treatments. For MDS/AA syndrome, the core or common denominator is a PIG-A mutated population that has undergone further mutation (PIG-A plus X). Rather than being separate diseases, they may be a part of a syndrome (Fig 14).

Patients with primary normo/hypercellular MDS or PNH are not part of the syndrome; however, all patients who develop hypoplasia/aplasia invariably belong to it, since their natural history includes PNH and/or MDS, whether AA was originally virus-, drug-, toxin-induced or idiopathic. A sequence of events leads to development of aplastic anemia/MDS and PNH was depicted in (Fig 15). In the first step a noxious agent (i.e. a virus, a toxic agent or a drug) causes transient HSC damage and induces repair involving hyperproliferation during which the incidence of PIG-A mutations is increased, since PNH clones are genetically unstable and disposed to further mutations. In the second step an additional genetic changes providing a growth advantage are likely to occur. This would lead to the presentation of an aberrant internal image on MHC molecules and thus to T-cell recognition and activation (Fig 15 A). Third step, the immune reaction against transformed HSC can be of three different types (Fig 15B, Nissen & Schubert, 2002).

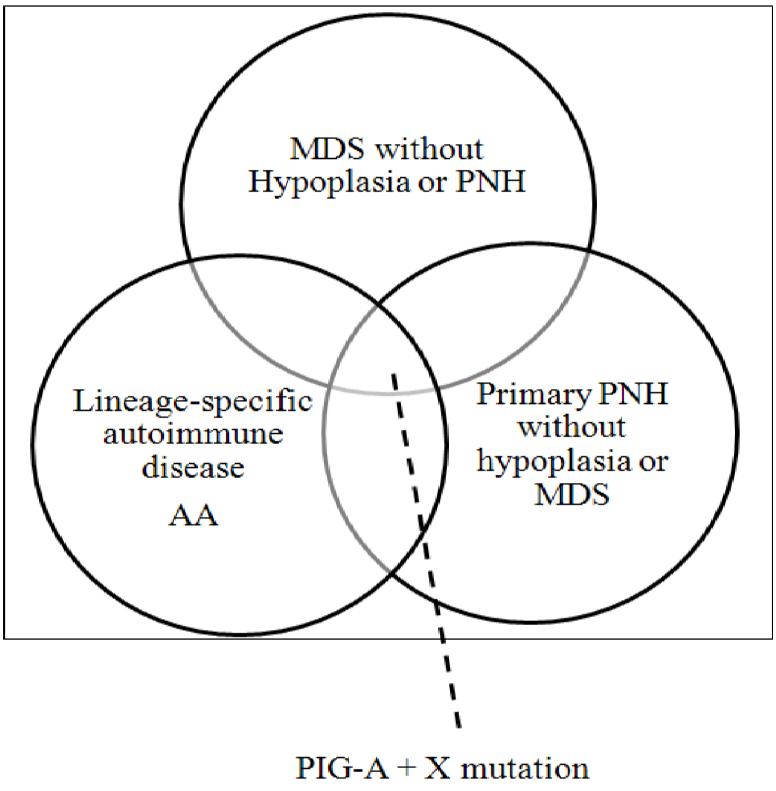
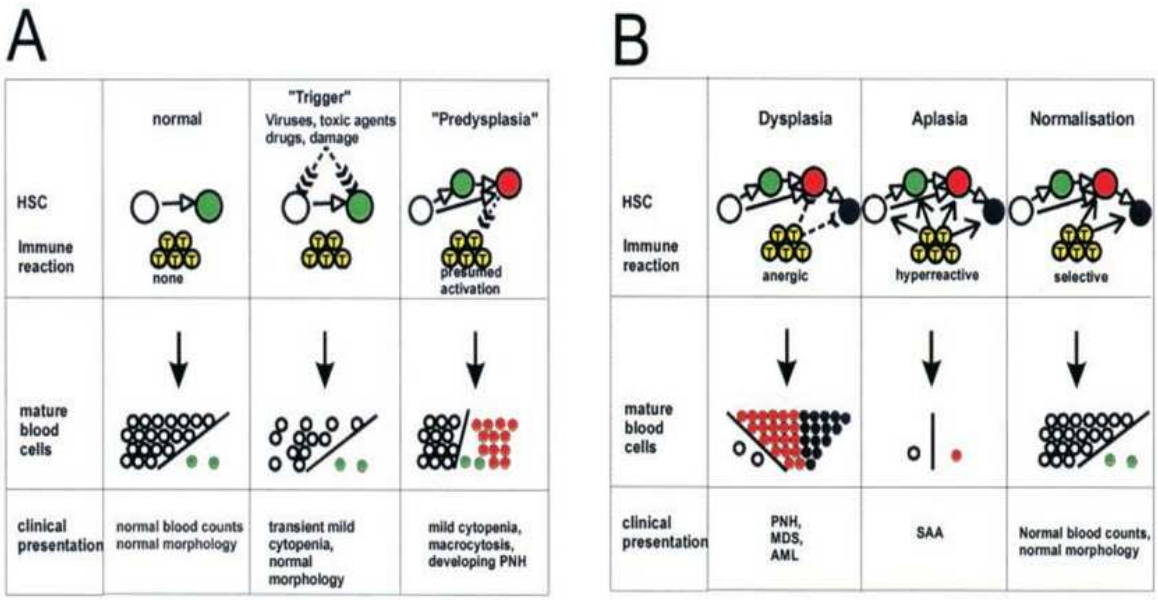


Figure 14: Proposed pathogenic link between aplastic anemia (AA), myelodysplasia (MDS) and paroxysmal nocturnal hemoglobinuria (PNH).



Normal cell (HSC), Cells with an isolated PIG-A mutation, PIG-A mutated cells with abnormalities conferring a growth advantage, Cells with additional cytogenetic abnormalities, T lymphocytes, Transformation route of HSC, Cytotoxic activity,

Putative T cell activation, Missing T cell activation.

Figure15: A hypothetical sequence of events leads to PNH or aplastic anemia, (A) HSCs with an isolated PIG-A-mutation occur in normal bone marrow. Exposure to toxic agents causes cellular damage and induces proliferative stress in HSC. PIG-A mutated cells or normal cells undergo mutations within genes involved in proliferation and differentiation leading to predysplasia. (B) Predysplastic cells alert the immune system, which reacts either with anergy, leading to expansion of abnormal clones, with a hyperreactive autoimmune reaction leading to severe aplastic anemia or with a selective immune reaction leading to cure. Modified according (Nissen & Schubert, 2002).

Hyporeactive/anergic response: In patients with a compromised immune system, the abnormal population expands and displaces normal HSC. PNH patients, though surviving long term with the abnormal clone have signs of immune incompetence: they are prone to infections and have low endogenous IL-2 production. Clinically, these patients present with PNH if the clone is stable and does not progress to leukemia or with MDS/AML if the clone undergoes further transformation.

Hyperactive response: In patients predisposed to autoimmunity, the immune reaction against the abnormal population extends to normal HSCs by the mechanism of ‘epitope spreading’. This causes severe aplasia with neither normal nor abnormal cells detectable in the BM. The more vigorous the immune reaction, the more acute and serious is BM failure and thereby the chance of transformed clones to be decimated. This immune attack puts pressure on mutated cells that attempt to survive by the development of new, potentially resistant clones.

Selective response: A specific immune reaction destroys the abnormal population, sparing normal HSC, leading to reconstitution of normal hematopoiesis. Clinically unrecognized expansion and subsequent immunological destruction of small potentially life-threatening cell populations is probably the most frequent event.

Murakami et al presented in 2002 (Murakami *et al*, 2002c) the first experimental evidence that supports the immunologic selection hypothesis for the clonal expansion of PNH cells and demonstrated that GPI^- hematopoietic cells become dominant in a mouse model on selection by allogeneic $CD4^+$ T cells. The study showed that GPI^+ APCs present the antigen derived from GPI-anchored proteins on MHC class II molecules, whereas GPI^- APCs do not. GPI-anchored proteins like other cell surface trans-membrane proteins would reach, via the endocytic pathway, a compartment where proteins are processed for presentation on MHC class II molecules. Proteins that are normally GPI anchored are not expressed on the surface of GPI^- APCs; hence, there is no presentation on the MHC class II molecules. They suggested that if the relevant autoantigen is derived from GPI-anchored proteins, PNH cells might be resistant to $CD4^+$ CTL (Figure 16A). They demonstrated also using in vitro systems that GPI^- APCs do not effectively stimulate antigen-specific and allogeneic $CD4^+$ T cells efficiently, which suggests that some GPI-anchored protein on APCs acts as a ligand for costimulatory molecules on T cells (Figure 16B, Murakami *et al*, 2002b).

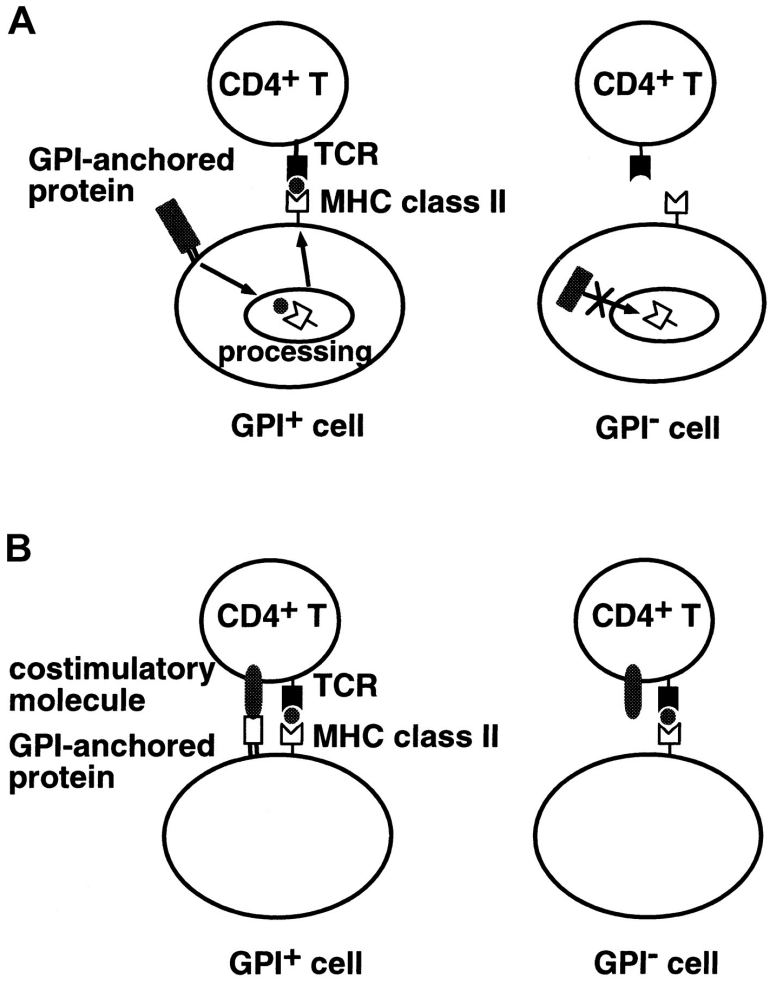


Figure 16: Two experimentally tested models. (A) A putative autoantigen recognized by $CD4^+$ T cells is derived from GPI-anchored proteins. GPI^+ cells (left) process GPI-anchored proteins and present antigenic peptides on MHC class II molecules; whereas GPI^- cells (right) do not present such antigenic peptides. (B) Some GPI-anchored protein is a ligand for some costimulatory molecules on $CD4^+$ T cells. GPI^+ cells (left) efficiently stimulate $CD4^+$ T cells; whereas GPI^- cells (right) do not. Modified according to (Murakami *et al*, 2002a).

5. Aim of the Study

The mechanism of clonal expansion of GPI-AP-deficient cells in PNH is still obscure. The fact that PIG-A mutation itself does not confer a proliferative advantage to HSCs suggests a second event to be responsible for clonal expansion of mutant cells. Two different hypotheses have been proposed to explain the clonal expansion in PNH, one assuming an immune escape mechanism and the other proposing an intrinsic second mutational event within clonal cells. In both hypotheses, the immune system supposed to play an important role. In the first theory (immune selection hypothesis), a defect in the immune system leads to immune attack directed against special antigens. GPI deficient cells can escape the immune system attack either by no expression of such antigens or by less susceptibility against cytotoxic attack of autoimmune T lymphocytes. In the second theory (clonal expansion through genetic alterations), the immune system recognizes the genetic alterations in target cells and produces antibodies against the modified antigens. Hence, the identification of these antigens will prove the occurrence of such alterations in the target cells. The identification of target antigens of the antibodies in PNH sera will give information about the pathophysiology of the disease and may give support to one or both theories. In MDS patients, the expansion of PNH-type cells was also reported and that phenomenon was linked in some studies to immunosuppressive therapy responsiveness. Identification of auto-antigens in MDS will demonstrate the immune mechanism of cytopenia in the disease. The aim of the study was to identify the target antigens of the immune system in PNH patients and MDS, study the serological reactivity of PNH patients, and compare it with that of healthy individuals, aplastic anemia, and MDS patients. Moreover, the genes that encode the target antigens in PNH were analyzed and the possibility of alterations of these genes by the patients was investigated.

V. Material and methods

1. Immunoscreening of cDNA expression library

Screening for candidate antigens was performed using the serological identification of antigens by recombinant expression cloning (SEREX) approach. This approach was first established by Pfreundschuh's group and applied for detection of tumor antigens in 1995 (Fig 17, Sahin *et al*, 1995). A human fetal liver cDNA expression library (Takara Bio Europe, Saint-Germain-en-Laye, France) was used as Antigens source. XL1-Blue Escherichia coli (Takara Bio Europe, Saint-Germain-en-Laye, France) was transfected with recombinant phages, plated on agar plates, and cultured at 37° C. Expression of recombinant proteins was induced by incubating the bacterial lawns with isopropyl β-D-thiogalactoside (IPTG) and the released proteins were Transferred to nitrocellulose filters (Hybond C-Extra; Amersham Pharmacia Biotech, Piscataway, NJ) by incubating at 37 °C for 90 minutes. Filters were then washed in TBST (10 mM Tris [tris (hydroxymethyl) amino methane], 150 mM NaCl, 0.05% Tween 20, pH 8.0) and blocked one hour with blocking buffer (5% wt/vol non-fat dry milk [Nestle, Solon, OH] in TBST). Filters were then incubated with patient sera diluted at 1:100. Specific binding of antibodies to recombinant proteins was detected by incubation with alkaline phosphatase-conjugated goat antihuman IgG antibody (Promega, Madison, WI) diluted at 1:2500. Visualization of the antigen-antibody complex was accomplished by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Promega). Complementary DNA inserts from positive clones were subcloned, purified, and in vivo excised to plasmid forms (Takara Bio Europe, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. The DNA inserts were subsequently sequenced with appropriate sequencing primers.

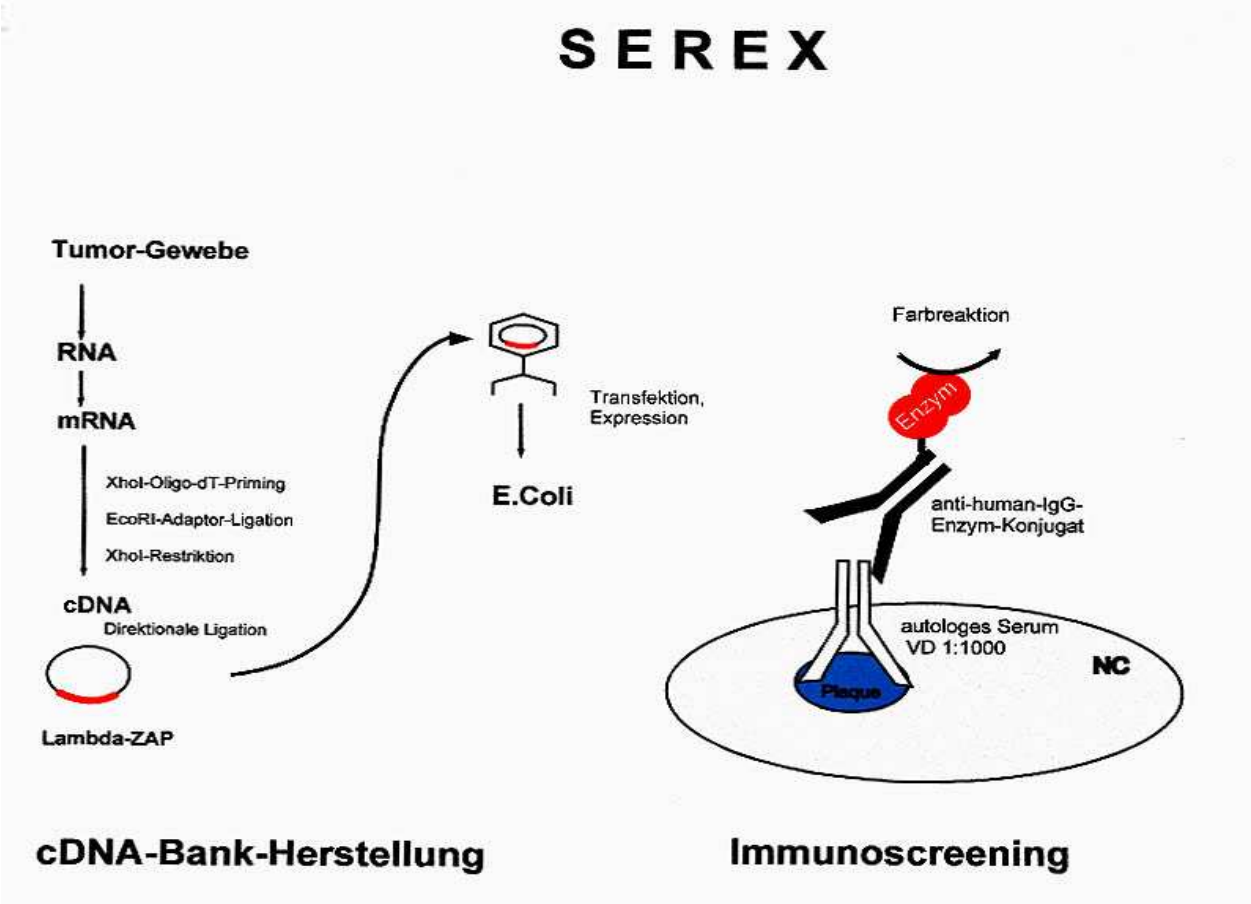


Figure 17: SEREX Method as described by Sahin et al (Sahin *et al*, 1997).

1.1. Preparation of patient's sera

Sera from 10 MDS patients were obtained at the Department of Hematology, Oncology and Clinical Immunology, Düsseldorf University Hospital (Düsseldorf, Germany). The sera were stored at - 80° C until use. The characteristics of all MDS patients are shown in table 4.

Table 4: Characteristics of MDS patients included in the study.

Patient number	Age and Gender	Hemoglobin level g/dl	Blood transfusion	Bone marrow	Diagnosis	
					IPSS classification	WHO classification
1	19 M	6.5	4/ month	Dysplasia in all 3 cell lineage, 4 % Blasts, hypocellular BM	Low-risk	RCMD
2	67 F	8.3	Unknown	Dysplasia in all 3 cell lineage, 4 % Blasts, hypocellular BM	Low-risk	RCMD
3	60 F	9.3	2/ month	Dysplasia in all 3 cell lineage, 9 % Blasts, hypocellular BM	High-risk	REB I
4	64 F	7.1	2/month	Dysplasia in all 3 cell lineage, 1 % Blasts, hypocellular BM	Low-risk	RCMD
5	65 M	8.7	Unknown	3 % Blasts, normocellular BM	Low-risk	MDS with del (5q)
6	63 F	9	Unknown	2 % Blasts	Not defined	MDS with del (5q)
7	40 M	7.4	No	6 % Blasts, normocellular BM	High-risk	RAEB I
8	64 M	6.4	Yes Unknown	1 % Blasts	Low-risk	MDS with del (5q)
9	61 M	7.3	Yes Unknown	1 % Blasts, normocellular BM	Low-risk	MDS with del (5q)
10	55 M	7.8	Yes unknown	15 % Blasts, hypocellular BM	High-risk	RAEB II

Sera from 10 patients with PNH were obtained during routine diagnosis or follow-up at the Department of Internal Medicine I, Saarland University Medical School (Homburg, Germany) and the Department of Hematology and Haemostaseology, University of Vienna (Vienna, Austria). Table 5 describes the characteristics of the PNH patients included in our study. Diagnosis of PNH had been established by flow cytometry.

Table 5: Characteristics of PNH patients included in the study.

Patient number	Sex/ age years	Hemoglobin level g/L	LDH* Units/L	Total Bilirubin mg/dl	Blood transfusion until the time of sampling	CD59- Erythrocytes %	CD 55- Granulocytes	CD 59- Granulocytes	Diagnosis	Therapy before the sampling
1	M45	8.6	2477	30.7	No	17.9	96.67	91.57	Classic-PNH	No
2	F 24	12.7	289	74.1	No	47.24	78.40	78.66	Classic-PNH	No
3	F 48	10.5	265	< 2	No	54.3	97.5	97.8	Classic-PNH	Eculizumab for 4 months
4	M56	10.1	263	2.5	+	85.9	90.2	88.1	Classic-PNH	Prednisone for 3 years
5	F 37	6.9	2735	53.4	++	83.31	94.52	93.12	Classic-PNH	No
6	F 53	10.2	313	0.48	No	11.50	25	84.94	Classic-PNH	No
7	M 61	11.5	476	1.61	No	No data	No data	No data	Classic-PNH	No
8	M 35	7.5	2504	5.82	+	30.92	97.35	98.03	Classic-PNH	No
9	M 66	9.3	1324	1.93	No	No data	No data	No data	Classic-PNH	No
10	M 69	12.7	272	51.9	No	43.05	1	0.8	Classic-PNH	Eculizumab for 4 years
(+) blood transfusion one a month, (++) blood transfusion two times a month. * LDH levels obtained at the time of blood taken for SEREX analysis										

Sera from five patients with newly diagnosed aplastic anemia (without PNH clones) were frozen before starting immunosuppressive therapy at the Institute for Transfusion Medicine and Clinics for Aplastic Anemia in Ulm (Ulm, Germany). Twenty healthy individuals donating blood at the Department of Transfusion Medicine, University of Saarland (Homburg Germany) were used as a control group. Patients gave informed consent for blood analysis. All sera were stored at - 80° C until use for screening as described below.

The same amount was taken from each of the 10 PNH sera and mixed together. These mixed sera were diluted 1:100 in 1×TBS containing 0.5% (wt/vol) low-fat milk. For preservation, 0.01% NaN_3 was added. These diluted sera were stored at +4°C and used for SEREX screening. In the same way, an AA pool, MDS pool and normal individual pool were made.

1.2. Preparation of host bacteria and transfection

The glycerol stock of XL1-Blue'MRF E.coli bacterium was streaked onto a Luria-Bertani (LB) tetracycline agar plate (tetracycline, 12.5 µg/ml) and the plate was incubated at 37°C overnight. Next day, a single colony of bacteria was picked from the LB agar plate and put into a 300 ml conical flask that contains 50 ml of LB medium supplemented with 0.2 % (w/v) maltose, 10 mM $MgSO_4$ and tetracycline (12.5 µg/ml). The bacteria were grown at 37°C for 4-6 hours under shaking, until the density of bacteria reached at an OD_{600} of 0.5. The cells

were spun down at 500×g for 10 minutes and the supernatant was discarded. The cells were suspended gently in 25 ml sterile water with 10 mM $MgSO_4$, diluted to an OD_{600} of 0.5 with sterile water containing 10 mM $MgSO_4$. The bacteria were stored at 4°C and ready for transfection.

1.3. Transfection with plasmid

600 µl XL1-Blue MRF' E.coli were incubated with appropriately diluted human fetal liver cDNA expression library (Takara Bio Europe, Saint-Germain-en-Laye, France) (4×10^3 recombinant phage/per 15 cm plate) at 37°C for 15 minutes to allow phage attach to the cells. Expression of fusion proteins in lytic phages was induced by adding 20 µl of 2 M isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 5 ml of top agar (pre-warmed at 52°C) to the bacteria and phage mixture. The top agar was quickly poured onto LB agar plate under carefully swirling in order to distribute the cells evenly. The plates were inverted and incubated at 37°C until plaques were visible (normally overnight).

1.4. Transfer of recombinant proteins onto nitrocellulose

When the plaques were grown to 1 mm of diameter on the plates, nitrocellulose membrane was placed onto each agar plate to absorb the protein from the plaques. A needle was used to prick through the membrane and agar with three asymmetric points for orientation. The plates were placed at 37°C for 1 hour, and then cooled at 4°C for 30 minutes before removing the nitrocellulose membranes from them. The membranes were washed in TBST (TBS containing 0.5% tween 20) for 10 minutes, under shaking. A piece of soft sponge was used to remove the residual agar from nitrocellulose membranes and the membranes were washed with TBST two times additionally, then blocked with 5% (W/V) low-fat milk in TBS at room temperature for at least 1 hour, and washed with TBS twice for 20 minutes.

1.5. IgG pretest

To exclude signals derived from the binding of secondary antibodies to expressed IgG-cDNA fragments that are included in the library, membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG Fcγ (Dianova, Hamburg, Germany) diluted 1:2.500 for 1 hour at room temperature. Subsequently, after washing in TBS twice under shaking, reactive phage plaques were visualized by incubating with 5-bromo-4-chloro-3-

indoly-phosphate (BCIP) and nitroblue tetrazolium (NBT) at 37 °C in dark condition for 10 to 30 minutes. A pencil used to mark signals.

1.6. Immunostaining

The membranes were incubated with 20 ml diluted sera (1:100) at 4 ºC overnight, under gently shaking. Next day, the sera were collected and the membranes were washed twice in TBS for 30 minutes. This was followed by an incubation with an alkaline phosphatase-conjugated goat anti-human IgG Fcγ (Dianova, Hamburg, Germany) diluted 1:250 for 1 hour at room temperature. After washing in TBS twice under shaking, reactive phage plaques were visualized by staining with 5-bromo-4-Chloro-3-Indolyl-Phosphate (BCIP) and nitroblue tetrazolium (NBT) at 37°C in dark condition for 5 to 10 minutes.

1.7. Isolation of positive clones

The stained membranes were checked carefully and the positions of reactive phage plaques were marked with a pencil. According to the marks, the reactive phage plaques were isolated from the original agar plates with sterile glass pipettes and transferred to a micro centrifuge tube containing 500 µl of SM buffer (NaCl, MgSO4,Tris pH 7.5, Gelatin) and 20 µl of chloroform. The tube was vortexed briefly and shaken for 1-3 hours at room temperature to release the phage particles into the SM buffer then storage at 4°C.

2. Sequence analysis of identified clones

The isolated phages from the original plate were diluted at different concentrations in SM buffer and the appropriate concentration was chosen to transfect bacteria. The procedures were performed as described above (see 1.3). The reactive clone was subcloned to monoclonality and submitted to in vivo excision of pTripelex vector and further serological assessment.

2.1. Conversion of the vector

The conversion of λTriplEx2 clone to a pTriplEx2 plasmid involves in vivo excision and circularization of a complete plasmid from recombinant phage. The plasmid is released because of Cre recombinase- mediate side-specific recombination at the loxP sites flanking the embedded plasmid (Figure 18).

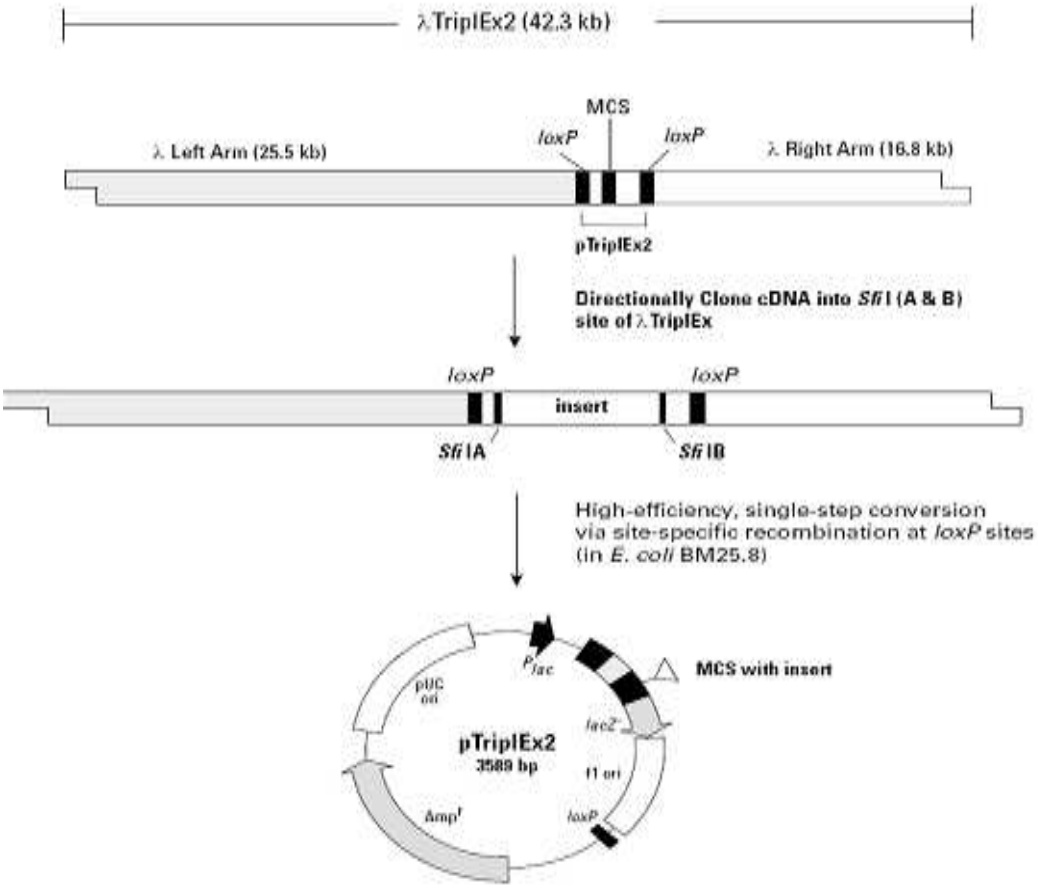


Figure 18: Conversion of a recombinant λTriplEx2 to the corresponding pTriplEx2. The λTriplEx2 MCS is located within an embedded plasmid, which is flanked by loxP sites at the λ junctions. Transduction of a λTriplEx2 lysate into E. coli strain BM25.8 promotes Cre recombinase-mediated release and circularization of pTriplEx2 at the loxP sites. pTriplEx2 carries the bla gene for ampicillin resistance and the pUC ori for autonomous replication in E. coli. The MCS provides several unique restriction sites flanking the SfiI A & B sites to facilitate the subcloning and analysis of inserts.

In this system, E.coli BM 25.8 provides the necessary Cre recombinase activity. The released version plasmid differs from the pTriplEx2 by a 100-bp loxP insert at the Cla site. The excised plasmid is propagated stably in E.coli. The process was done in the following steps:

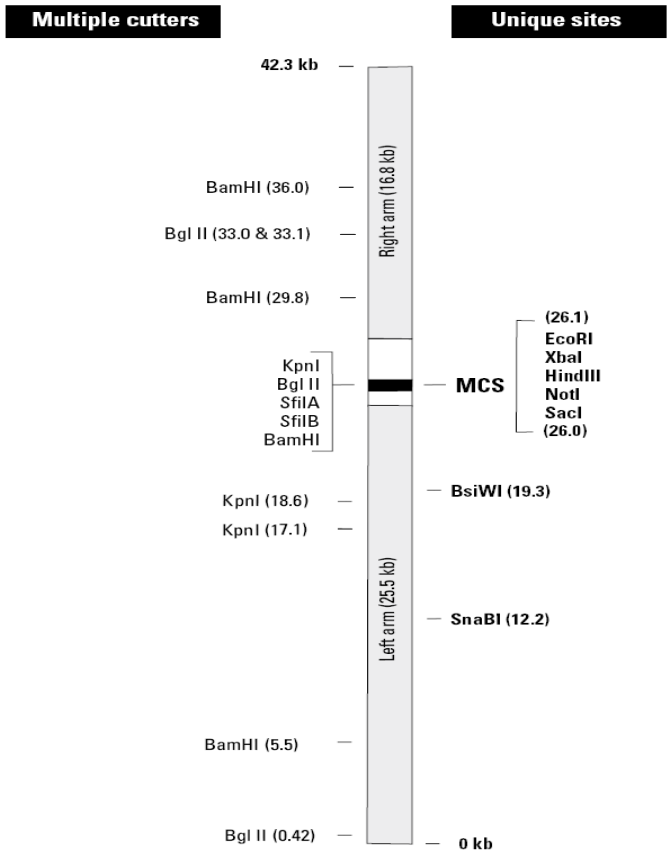


Figure 19: Restriction Map of λTriplEx2.

Single, isolated colonies from the working stock plate of BM25.8 host cells were picked and used to inoculate 10 ml of LB broth in a 50-ml test tube or Erlenmeyer flask. The culture was incubated at 31°C overnight with shaking (at 150 rpm) until the OD600 of the culture reached (1.1–1.4). After that, 100 µl of 1 M MgCl2 were added to the 10-ml overnight culture of BM25.8 (10 mM final concentration of MgCl2). A well-isolated positive plaque from secondary- or tertiary screening plates was picked and placed in 350 µl of 1M lambda dilution buffer. After vortexing the plaque, it was incubated at 37°C for 3–4 hours without shaking (200–250 rpm). Then, in a 20-ml test tube: 200 µl of overnight cell culture was combined with 150 µl of the eluted positive plaque. The Mixture was incubated at 31°C for 30 min without shaking then 400 µl of LB broth was added and incubated at 31°C for an additional 1 hr with shaking (225 rpm).

Using a sterile glass spreader, 1–10 µl of infected cell suspension was spread on an LB/carbenicillin (or Ampicillin) plate to obtain isolated colonies and incubated at 31°C overnight. Several well-isolated colonies from each clone were picked and prepared plasmid

DNA separately from each one. The isolated plasmid DNA should be pure enough for direct sequencing. Provided pTriplEx2 sequencing primers used with standard Ds-DNA-sequencing protocol.

2.2. Restriction enzyme digestion of plasmid DNA

Because the high quality of sequence data depends directly on the quality and quantity of the template DNA, it is important to make a restriction digest of the plasmid DNA and evaluate the size and the quantity of the cloned DNA insert. The mixture of enzyme reaction contains 1 µl of plasmid DNA, 0.5 µl of EcoRI and Xho I enzyme (Fermentas, 10U/µl), 4 µl of 10×buffer (Y+/TanqoTM, Fermentas), and H2O to the final reaction volume of 20 µl. The reaction was incubated at 37°C for 3 hours. To check the progression of a restriction enzyme digestion, 1% agarose gel with ethidium bromide in 1×TAE buffer was prepared. Digested plasmid DNA and DNA markers were loaded onto the gel. Electrophoresis was performed at 80 V for 1 hour. After electrophoresis, the gel was placed on an UV light box and a picture of DNA separation pattern was taken with a Polaroid camera.

2.3. Determination of nucleotide sequence

2.3.1. Sequencing reaction

Double strand plasmid DNA with insert was sequenced in both directions by the dideoxynucleotide termination method (Sanger *et al*, 1977). The four reactions mixes were set up for each template, respectively, each containing all four deoxynucleotides triphosphates (dNTPs) and one of the four dideoxynucleotide triphosphates (ddNTPs), to generate four different sets of fragments. The synthesis of DNA strand is initiated at a vector specific site to which the primer is annealed and terminated by the incorporation of a ddNTPs.

The sequence reaction was performed using the SequiTherm EXCELTM DNA Sequencing Kits-LC (Epicentra Technologies, Madison, WI) with fluorescent-labeled primers. The procedure was done as follows: for each template, 2 µl of each termination mix G, A, T, C was dispensed into four different microcentifuge tubes labeling with G, A, T and C. The master mixture was prepared by mixing 7.2 µl of sequence buffer, 0.5 µl of each labeled primer (2 pmole, IR41-labeled reverse and universe primer), 50-250 fmoles of DNA template, 1 µl of DNA polymerase (5 u/µl) and sterile, deionized H2O to bring total volume to 17 µl. Followed by dispensing 4 µl of the mixture to each of four tubes containing Termination Mix (G, A, T, C), respectively. A drop of mineral oil was placed on top of each reaction mixture,

and tubes were put into a TRIO-Thermoblock (Biometra, Göttingen, Germany). Sequencing reaction was started at 94°C for 5 minutes to denature double-stranded templates. Then 30 cycles were performed, during each cycle, the reaction was raised to 94°C for 1 minute to denature double-stranded templates, lowered to 60°C for 1 minute to anneal the primer to the template, and then raised to 70°C for 1 minute for the elongation step. When the PCR was finished, 4 µl of stop / loading buffer was added to each reaction tube.

2.3.2. Denaturing polyacrylamide gel electrophoresis

The denaturing polyacrylamide gel was prepared in 0.2 mm thickness using a set of 41 cm long glass plates. The gel contained 30 ml of Sequagel XR (6% National Diagnostics, BIOZYM), 7.5 ml of Sequagel buffer, 400 µl of DMSO (Sigma) and 300 µl of 10% fresh APS. After the gel had been cast, it was allowed to polymerize for 1 hour at room temperature. Then, the gel was installed in a LICOR automatic sequencer (Model 4000L) following pre-electrophoresis with 1×TBE buffer at a running condition of 1500 V, 37 mA and 50°C for 45 minutes. The loading area of the gel was rinsed carefully by pipetting gently up and down and a shark toothcomb was inserted until the teeth just touched the gel, with the teeth forming the sidewalls of the wells. In order to separate the synthesized DNA fragment from the template strand, and to allow the DNA to flow through the gel before loading samples onto the gel, the products of sequencing reaction have to be denatured by heating at 80°C for 5 minutes and chilled on ice immediately. 1 µl product of each reaction was loaded into the wells of the gel according to G, A, T, C in adjacent order. The sequencing reaction products are size-separated by the denaturing polyacrylamide gel electrophoresis. Sequence alignments were collected and analyzed with DNASIS (Pharmacia Biotech, Freiburg, Germany) and BLAST software on NCBI GenBank (Altschul *et al*, 1990).

3. Serological analysis of positive clones

3.1. Preparation of sera

To remove antibodies in the serum that could react with bacterial or phage components the serum used for serological analysis must be pre-absorbed by the following two methods. This was only done for sera that were used at high concentration (dilution 1:100).

3.1.1. Affinity chromatography absorption

Antibodies to E. coli and phage components can be removed from serum using affinity chromatography that exploits the specific binding of antibody to these antigens held on a solid matrix. These antigens are bound covalently to small, chemically reactive beads loaded into a column. When serum passes over the column containing the antigen binding beads, antibodies to E. coli phage components will bind to the beads on the column, which results in the removal of antibodies against bacteria and phages from the serum. In this study, two different affinity columns were used for the treatment of the sera.

a) Preparation of L column

The L column contains bacteria and phage components. For the preparation of the L column, 50 ml of LB-Tetracycline-medium containing XL-1 blue MRF' E. coli is cultured at 37°C with shaking overnight. Aliquots of the bacterial culture medium were placed in a 50 ml tube and centrifuged at 3000 rpm for 30 minutes to save the pellet. The bacterial pellet was then resuspended with 2 ml of 0.01 M MgSO4, of which, 200 µl of insert-free λTriplEx2 phages in 5 ml of LB-medium. The mixture of phages and bacterial was placed at 37°C for 4 hours under shaking for lytic infection. The rest of the bacterial suspension mixed with 5 ml of LB-medium was added to this cultured mixture to continue incubation for another 2 hours at the same conditions as before. Then, the mixture was centrifuged at 8000 rpm for 10 minutes. The pellet was saved and resuspended in 10 ml of 1 × MOPS buffer (3-(N-Morpholino)-propanesulfonic acid) followed by sonication for 10 seconds twice on ice. The disrupted bacteria were mixed with 1 ml of affinity adsorbent (Roche Diagnostics GmbH, Mannheim, Germany) in a 50 ml Falcon tube. The tube was agitated by overhead rotation at 4°C overnight in order to let the affinity adsorbent bind the antigens completely.

After overnight rotating, the tube containing the antigen binding absorbent should be rinsed in 20 ml of PBS containing 0.01% NaN3 by rotating at 4°C for 30 minutes. After discarding the PBS, 20 ml of 1 M glycine is loaded into the tube to continue rotating for 2 hours at 4°C. Finally, the PBS wash is repeated once more. The tube is ready for pre-absorption of serum.

b) Preparation of M column

The M column contains only bacteria antigens. The pellet of XL-1 blue MRF' was directly resuspended in 10 ml of 1×MOPS buffer by vortexing. The bacteria were disrupted by

sonication and mixed with affinity adsorbent. The procedure is the same as that described above for the preparation of the L column.

3.1.2. Lytic bacteria membrane absorption

Empty pTriplEx2 phages (pfu 50,000) (without cDNA inserts) were incubated with XL-1 blue MRF' (OD600=0.5) at 37°C for 15 minutes, followed by mixing with top agar without adding IPTG and plating on a LB-Tet agar plate. After incubating overnight at 37°C, the phages were blotted onto a nitrocellulose membrane and kept at 37°C for 1 hour. The membrane was then blocked with 5% low-fat milk and subsequently washed. The main procedure was the same as described above. The membrane containing the lytic bacteria was air-dried, stored at room temperature and was ready to use for the pre-absorption of the sera.

3.1.3. Pre-absorption of sera

The sera were first diluted at 1:50 with 20 ml of 0.5% low-fat TBS and loaded to the M columns with rotating overnight at 4°C. Each serum was transferred from the M to the L column to continue absorption under the same conditions. After passing through both columns, the serum was placed in a plate with a lytic bacterial membrane by shaking at room temperature. A new lytic bacterial membrane replaced the lytic bacterial membrane every 4 hours for four times. Finally, the serum was diluted to 1:100 containing 0.01% NaN3 and Thimerosal. The serum was stored at 4° C until use. After passing of the serum, the column was regenerated by rinsing in acid condition in order to elute the binding antibodies. 30 ml of 0.1 M Tris (pH 3) was used to wash the column thrice for 30 minutes at 4°C with rotating, followed by a wash in TBS twice 30 minutes. The column can be preserved in PBS containing 0.01% NaN3 in 4°C for reuse.

3.2. Phage assay for the detection of serum antibodies

For the detection of antibodies with binding activity for the proteins in the lytic plaques, sera from different individuals and monoclonalized phages from the positive clone were mixed, at a ratio of 1:10, with non-reactive phages of the cDNA library as an internal negative control. To avoid false negative results due to the decomposition of sera, a clone, PINCH (EMBL Data library, Accession No.U09284) known to react with most of human sera, was chosen as a positive control for serum quality. The clone for positive control and the clone positive in the library screening were used to transfect bacteria, respectively. Then, transfected bacteria were mixed with top agar and IPTG by plating on separate quarters of the same agar plate.

The plate was incubated in 37°C overnight, followed by blotting onto a membrane and serially washed. The 1:100 diluted E coli-absorbed sera were tested with immunoscreening procedure as described above. To compare different serum relativities, testing was done in parallel and developing was stopped after 5 minutes.

4. Expression assay by reverse transcription PCR

4.1. Hematopoietic progenitor cell assay

$CD34^+$ cells were purified from bone marrow of PNH-1 (which was positive against the first isolated clone) and a non PNH patient (as control) using magnetic cells sorting (MCS Kit-Miltenyi Biotec , Germany) according to the manufacturer's instructions.

4.1.1. Preparation of bone marrow cells

Bone marrow was collected in 50 mL tubes containing 5 mL PBS + 2 mM EDTA. For preparation of single cell suspension of bone marrow cells, it was diluted with 10 x the volume of RPMI 1640 containing 0.02 % collagenase B and 100 U/mL DNase and was shaken gently at room temperature for 45 minutes. The cells were passed through 30-µm nylon mesh (Miltenyi Biotec, Germany); the mesh was wetted with buffer before use. 35 mL of diluted cell suspension was layered carefully over 15 mL of Ficol-plaque. It was centrifuged for 35 minutes (400 g, +20˚C) in swinging-bucket rotor without brake. The upper layer was aspirated leaving the mononuclear cell layer undisturbed at the interphase. The interphase cells (lymphocytes, monocytes and the thrombocytes) were carefully transferred to a new 50 mL tube. The tube was filled with PBS containing 2 mM EDTA and centrifuge for 10 minutes (300g, 20˚C). The supernatant was removed completely, and the cell pellet was resuspended in a final volume of 300 µl of Buffer for up to 10^8 total cells.

4.1.2. Magnetic labeling

The number of cells was determined using a Neubauer counting chamber, the cell suspension was centrifuged at 300g for 10 minutes, and the supernatant was aspirated completely. The cell pellet was suspended in 300 µl of buffer up to 10^8 total cells and 100 µl of FCR blocking reagent was added for up to 10^8 total cells then 100 µl of CD34 Microbeads for up 10^8 total cells. Mixed well and refrigerated for 30 minutes (4-8˚C).

After that, the cells were washed using 5-10 mL of buffer for up to 10^8 cells, centrifuged at 300 g for 10 minutes. The supernatant were completely aspirated and the pellet was resuspended up to 10^8 total cells in 500 µl of buffer.

4.1.3. Magnetic separation with LS columns

A column was placed in the magnetic field of a suitable MACS Separator. The column was prepared by rinsing with appropriate amount of buffer (LS: 3 mL). The cell suspension was applied onto the column. Unlabeled cells that pass through were collected and the column was washed with appropriate amount of buffer. The washing steps were performed by adding buffer three times. A new buffer was added only when the column reservoir is empty (LS: 3X3 mL). The total effluent was collected; this is the unlabeled cell fraction.

The column was removed from the separator and placed on a suitable collection tube. An appropriate amount of buffer (LS: 5 mL) was added onto the column and the magnetically labeled cells were immediately flushed out by firmly pushing the plunger into the column. The number of cells was determined again and proceeded to RNA extraction.

4.2. Extraction of Total RNA

Ribonucleases are very stable enzymes responsible for RNA hydrolysis. RNase A can easily survive autoclaving and other standard methods of protein inactivation. To prevent contamination of RNase, all glassware used for RNA extraction should be baked for a minimum of 3 hours at 250°C. For plastic ware, RNase-free products should be used or be treated with DEPC-H2O (de-ionized water treated with diethyl pyrocarbonate) overnight and autoclaved for at least 30 minutes. H_2O should be treated with 0.1% DEPC (Diethylpyrocarbonate, Sigma) overnight under shaking and autoclaved to remove residual DEPC.

Total RNA was isolated by a modification of the single-step acid guanidium thiocyanate (GITC)-phenol-chloroform RNA extraction method (Chomczynski & Sacchi, 1987) or by using the RNeasy Mini Kit (Qiagen, Germany). Integrity of the RNA was checked by electrophoresis, the yield was measured by UV absorption. 5 ml of GITC-buffer was mixed with 1% β-mercaptoethanol (Sigma) in a 12 ml tube (Falcon, “white caps”) and the mixture was added to about 500 µl cell suspension, and immediately homogenized until a completely homogeneous lysate was obtained. Then 0.5 ml of 2 M sodium acetate (pH 4.0), 1 ml of chloroform and 4.5 ml of TE-saturated phenol pH 4.5 (Carl Roth GmbH, Karlsruhe,

Germany) were added. The mixture was vigorously vortexed and placed on ice for 30 minutes. To get the phase separation, the mixture was centrifuged (7500g, 4°C) for 15 minutes and 4 ml upper aqueous layer was carefully transferred to a new tube containing 6 ml of isopropanol. After vortexing briefly, the sample was placed at -20°C overnight. A subsequent wash with cold ethanol (80%) followed by brief centrifugation. The RNA pellet was air dried at room temperature for 15-30 minutes, and dissolved in DEPC-treated H2O. Aliquots of RNA were stored at - 80 °C until use.

For checking the integrity and assessing the amount of RNA, 1µl of RNA mixed with loading buffer was placed at 70 °C for 5 minutes and quickly chilled on ice. The sample of RNA and a standard of RNA (E. coli RNA) used to evaluate the amount of RNA were loaded in 1% formalin/MOPS gel. Electrophoresis was performed with 1×MOPS buffer at 80V for 40 minutes. RNA pattern was visualized under the UV light.

4.3. First-strand cDNA synthesis

For each reaction, 2-5 µg of total RNA was mixed with 1 µl of dT18-oligonucleotide (50 pmol/µl), 1 µl of dNTP (10 mMol of each dATP, dTTP, dCTP, dGTP) and DEPC-treated H_2O to a volume of 13 µl. The mixture was heated to 70°C for 5-10 minutes and quickly chilled on ice.

The content of the tube was collected by brief centrifugation and 6 µl of the master mixture was added containing 2 µl of 0.1 M DTT, 4 µl of 5 × first-strand cDNA buffer and 0.8 µl of moloney murine leukemia virus (MMLV) reverse transcriptase II (GIBCO/Life Technologies), followed by pipetting gently up and down for mixing. The reaction was incubated for 60 minutes at 42°C, and then the reaction was stopped by heating at 70°C for 15 minutes. The entire process was performed in a programmable thermocycler (Biometra, Germany). The first-strand cDNA was stored at -20°C until use.

Integrity of the cDNA was proven by amplification of ubiquitously expressed p53 gene transcripts in a 30-cycle PCR (Gure *et al*, 2000), using the following primers: 5'-ACT GAA CAA GTT GGC CTG CAC-3' (sense, exon 10) and 5'-TGC AGA TGT GCT TGC AGA ATG-3' (anti-sense, exon 11). Only those cDNA, that expressed p53 well, were used further.

4.4. RT-PCR analysis of identified clones

To evaluate the mRNA expression pattern of identified clones, RT-PCR was performed by amplification of the cDNA from normal and PNH $CD34^+$ cells. The gene-specific primers utilized in RT-PCR reactions were designed to amplify the coding sequences of corresponding serologically defined antigens and synthesized commercially (MWG, Biotech).

For MPHOSPH 1, the forward primer (5`- GTGAGTAAAAATGCTCTCAG -3`) and the reverse primer (5`- GAAGCTGACTTTAAGAC -3`) were applied. For desmoplakin, the forward primer 5`- GGCTTCGAGGGTGTGAAGGGAAAGAA -3`) and the reverse primer (5`- TGCGGTGTCCCTTAAGAAGGATGA -3`) were used.

0.8 µl of first-strand cDNA was amplified using 0.2 U AmpliTaq Gold (Perkin Elmer, Weitersadt, Germany), 1 µl of dNTP (10 mMol of each dATP, dTTP, dCTP, dGTP), 1 µl of each primer and 3 µl of PCR buffer in a reaction volume of 30 µl. The amplification was performed with 35 cycles at a denaturation temperature of 94°C (1 min/cycle); an annealing temperature of 60°C for desmoplakin and 51°C for MPHOSPH 1(1 min/cycle); and an extension temperature of 72°C (1 min/1kb). The PCR products were analyzed by agarose gel electrophoresis. GAPDH was used as Housekeeping gene, and Testis cDNA as DNA positive control.

4.5. Electrophoresis procedure

The electrophoresis chamber was filled with (1x TAE) buffer. Each PCR-product was mixed with blue marker 5:1 (i.e. 10 µl PCR product + 2 µl marker) in a clean Eppendorf tube. The rest of the PCR product was stored at 2-8ºC until use. Each mixture was pipetted into one of the 1% agarose-gel cups. In the first cup, 10 µl of DNA marker VIII (Roche, Germany) was added. The agarose-gel was placed in the electrophoresis-chamber and the electrophoresis was performed using 400 mA, 100V for 45 min. After that, the agarose-gel was exposed to UV-light (UV-photo documentation) to visualize the products.

5. Study of antibody levels in the sera of PNH patients and controls

5.1. Generation of MPHOSPH 1 expression construct

5.1.1. Amplification of MPHOSPH 1 construct with PCR

MPHOSPH1 cDNA was amplified from isolated phages using PCR with the following primers: forward primer (5`- GATATCATGCACAGCATATTCACTGTTAAA -3`) and reverse primmer (5`- GATATCACTTTGAAAATAGTGAGTCAGAAA -3`)

Both primers were diluted with appropriate volume of distilled water to get the concentration of 100 pmol/ µl, after that they were mixed 1:1 and stored at -20 ºC until use. This stock solution was diluted 1:10 with distilled water when used. The following PCR components were mixed in an Eppendorf: 0.5 µl DNA (isolated phages), 2 µl primers mix, 0.2 µl dNTPs, 3 µl Taq buffer, 0.2 µl Taq polymerase and 24.5 µl distilled water. 0.5 µl water was used instead of DNA as negative control, the amplification was performed using the program showed in table 6:

Table 6: PCR program used to amplify MPHOSPH1 cDNA.

	Time (min: sec)	Temperature (ºC)	Cycle number
Hot start	12:00	94	1
Denaturation	01:00	94	35
Annealing	01:00	51	
Extension	01:00	72	
Final extension	9:00	72	1

After amplification, the DNA product was mixed with blue marker and separated according to the molecular weight using gel electrophoresis on 1% agarose gel for 45 minutes. The next step was to detect the products using ultra violet light source and by comparing with the used DNA-marker VIII the position of MPHOSPH1 band was determined (~ 300 bp).

5.1.2. DNA cloning with TA vector

The plasmid vector pCRII-TOPO (Invitrogen, Karlsruhe, Germany) was used for the first step of cloning. Because the vector is supplied linearized and contains single 3´-thymidine (T) overhangs for TA Cloning and topoisomerase I covalently bound to the vector, the PCR product amplified with Taq polymerase could be cloned directly. In an Eppendorf, 0.5 µl PCR product was added to 1 µl salt solution, 0.5 µl vector and 3 µl distilled water. It was mixed gently and incubated for 5 minutes at room temperature (22-23°C). After the incubation time, the reaction was placed on ice.

5.1.3. Transformation into DH5α bacteria

Transformation of the cloned product leads to a multiplication of the product depending on the multiplication of the bacteria. Transforming into DH5α (Invitrogen, Karlsruhe, Germany) was done using the manufactures guidelines as following: 2 µl of the TOPO cloning reaction was added into a vial of (1 µl) DH5α and mixed gently. The mixture was incubated on ice for 5 to 30 minutes. After that, the cells were exposed to Heat-shock for 30 seconds at 42°C without shaking. The tubes were immediately transferred onto ice and kept stay for 5 minutes. 10- 50 µl of cells were spread on a pre-warmed LB plate containing 50-100 µg/ml ampicillin and incubated overnight at 37°C.

5.1.4. Analysis of Positive Clones

Tens Miniprep protocol (GE Healthcare, Munich, Germany) was used to isolate the plasmid DNA from DH5α; the following materials are necessary for the isolation:

TENS Buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
0.1 N NaOH
0.5% SDS
TE pH 8.0
10 mM Tris-HCl pH 8.0
1 mM EDTA pH 8.0
95% EtOH precooled to -20 º C
70% EtOH precooled to -20 º C
3 M Na Acetate pH 5.2
RNAse A

The isolation was done using the manufactures guidelines: 2-6 colonies were taken and cultured overnight in 4 ml LB medium containing 50 µg/ ml ampicillin.1.5 mL of culture was centrifuged at 14.000 rpm for 1 min and the supernatant was removed. To the Pellet, 20 ug/ml RNase A was added and vortexed for 5 seconds. After that, 300 µl of TENS was added and vortexed for 5 seconds. After adding 150 µl of 3M NaOAc pH 5.2 and well mixing, a white precipitate was formed. The supernatant was transferred by pouring into a fresh tube and washed with 1 ml 95% EtOH and spined at 14 krpm for 1 minute. The supernatant was removed. The pellet was washed with 1 ml 70% EtOH and dried in air for 5-10 minutes at RT. After that, it was resuspended in 30 µl distilled water.

In an Eppendorf, 2 µl DNA was mixed with 4 µl 10X Tango buffer (Fermentas GmbH, St. Leon-Rot. Germany), 0.5 µl enzyme (Eco RI) and 13.7 µl distilled H2O. The mixture was incubated at 37°C for 60 min, and then run on 2% agarose 1xTAE gel to determine the clones containing the required inserts.

5.1.5. Cloning with pSFiExpress-HA

MPHOSPH1 insert was subcloned in frame into pSfi-Express-HA vector (Fig 20) for His-tag fusion protein. pSfi-Express-HA vector is a derivative of pEGFP-C1 vector (Takara Bio Europe, Saint-Germain-en-Laye, France), the plasmid has a neomycin resistance gene for selection in mammalian cells. At first, both insert and vector were digested with SMAI restrict Enzyme (Fermentas GmbH, St. Leon-Rot. Germany). The digestion was done as following:

In an Eppendorf; 5 µl Insert or 1 µl vector, 5 µl 10X buffer (tango), 0.5 µl BSA, 39.5 µl distilled H2O and 0.5 µl enzyme SMAI were mixed well.

The mixture was incubated at 37°C for 120 min and run on 2% agarose 1xTAE gel. Two bands could be identified. The first band was about 2000 bp, which referred to the vector, and the second band was about 300 bp for MPHOSPH1-DNA.

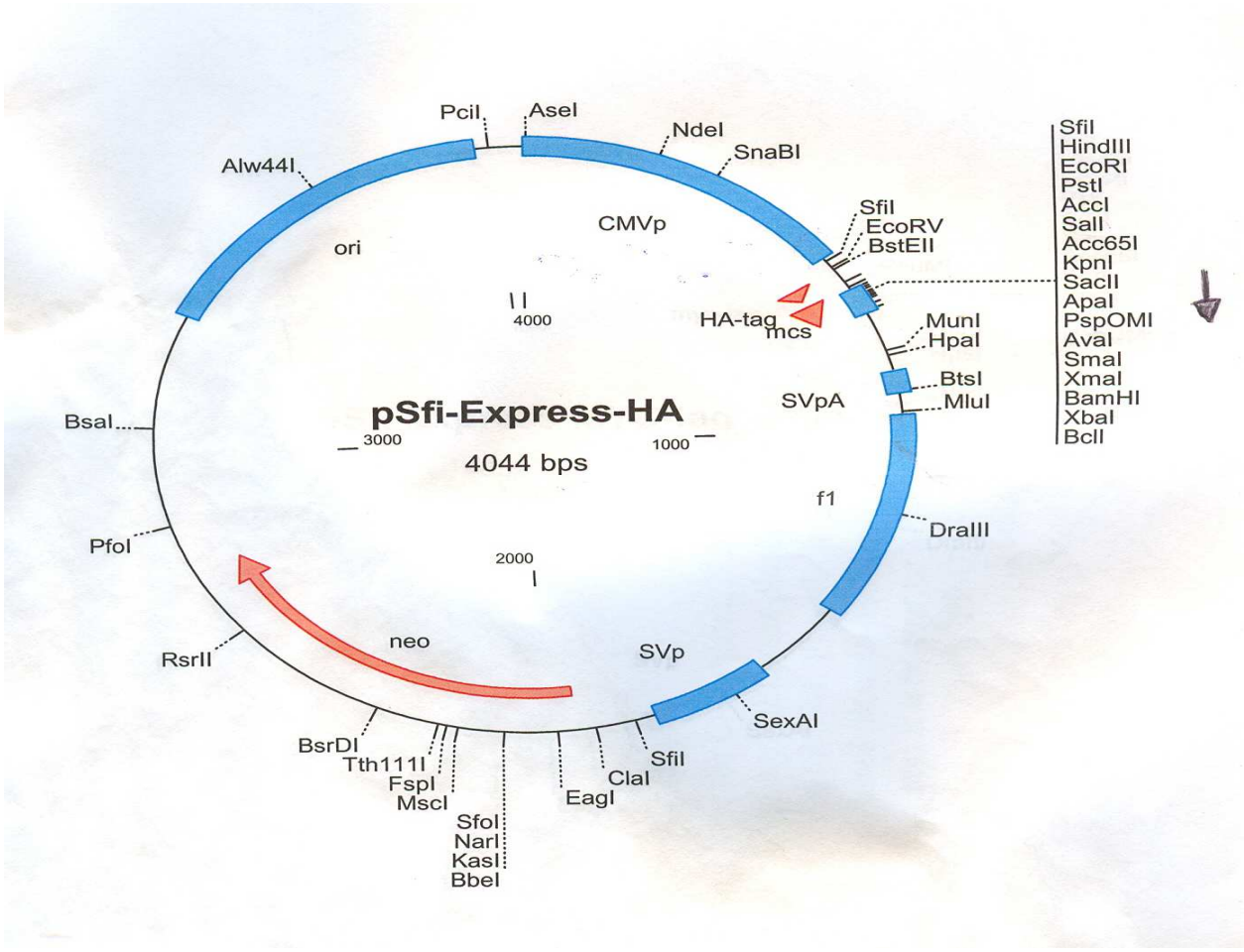


Figure 20: A schematic representation of pSfi-Express-HA vector.

5.1.6. DNA purification from Gel Band

GFX PCR DNA and Gel Band purification kit (GE Healthcare / Germany) was used to purify the construct from agarose gel, the kit uses a chaotropic agent that denatures protein, dissolves protein, and promotes the binding of double- stranded DNA to a glass fiber matrix. Once the DNA is captured, protein and salt contaminants are washed away, and the purified DNA is eluted in a low ionic strength buffer (TE, Tris-HCl or water). The following components were included in this kit:

Capture buffer	Buffered solution containing acetate and chaotrop
GFX columns	MicroSpin columns pre- packed with a glass fiber matrix.
Collection tubes	2 mL capless microcentrifuge tube.
Wash buffer	Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Add absolute ethanol to a final concentration of 80 % before use.

The following reagents were necessary and not supplied in the Kit: absolute ethanol and Elution buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA or TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The following equipments were necessary for the purification:

- Microcentrifuge: that accommodates 1.5 ml microcentrifuge tubes.
- Tubes: 1.5 ml microcentrifuge tubes.
- Scalpel or razor blade.
- Incubator or water bath / 60º C.

The purification was done according to the manufacturer's instructions as follows: an empty 1.5 ml microcentrifuge tube was weighed and the weight was recorded. Using a clean razor blade or scalpel, the slice of agarose containing the DNA band was excised to be purified and the slice was cut into several smaller pieces then transferred them to the pre-weighted 1.5 ml microcentrifuge tube. The tube was weighed again and the weight of the slice was determined by subtracting the weight of the empty tube. Then, 10 µl of capture buffer was added to each 10 mg of gel slice (maximum column capacity is 300 µl of capture buffer added to 300 mg gel slice). After closing the tube, the content was mixed by vortexing vigorously and incubated at 60º C until the agarose is completely dissolved (5-15 min). One GFX column for each tube was placed, during the incubation in a collection tube. After the agarose had been completely dissolved, it was briefly centrifuged to collect the sample at the bottom of the tube. The sample was transferred to the GFX column and incubated at room temperature for 1 min. After that it was centrifuged in a microcentrifuge at full speed for 30 s, the flow-through was discarded by emptying the collection tube, and the GFX column was placed back inside the collection tube. The DNA was washed by adding 500 µl of wash buffer to the column and it was centrifuged at full speed for 30 s. The collection tube was discarded and the GFX column was transferred to a fresh 1.5 ml microcentrifuge tube. After that, a 10µl double distilled was applied water directly to the top of the glass fiber matrix (in the GFX column) and incubated the sample at room temperature for 1 min. To recover the purified DNA, the tube with column was centrifuged at full speed for 1 min then it proceeded to ligation step.

For the cloning of MPHOSPH1-DNA with pSfi-Express-HA vector, the following reagents were placed into a 1.5 ml tube: 2 µl DNA + 10 µl ligase buffer + 1 µl pSfi-Express-HA vector + 2 µl DNA ligase.

The DNA-ligase enzyme ligates then the two cleaved ends of the vector. The ligation step was done at 16 ºC over night.

Transformation and miniprep were done as mentioned above. (See paragraph 5.1.4)

5.1.7. Digestion with enzymes

In order to determine which of the colonies contains the MPHOSPH1-DNA fragment, the fragments were digested using appropriate restriction enzymes. Using the NEBcutter V2.0 website (http://tools.neb.com/NEBcutter2/index.php), the enzymes that do not cut MPHOSPH1 sequence was selected.

Two enzymes were used here because using one enzyme will cleave the vector in one site, leading to a linear DNA with almost the same size. When two enzymes are used, the vector is cleaved in two different sites into two pieces. These two DNA fragment differ in size depending on whether the vector include the insert or not.

Two different enzymes (Hind III and NdeI) were incubated for 60 min at 37ºC with the extracted DNA product from the last step. The following materials were used for this step: 1µl BglII, 1µl EcoRI, 2 µl Orange buffer, 0.5µl BSA, 3 µl DNA (from miniprep), 10.5 µl H2O. The products were then mixed with blue marker and an electrophoresis on 2% agarose gel was performed. According to the molecular weight of the bands viewed by UV-light, the colony, which contained the proper DNA fragment, could be determined.

5.1.8. Preparation of Midi preps

QIAGEN Plasmid Midi kit (QIAGEN- Dusseldorf, Germany) was used in order to gather enough plasmid DNA for transfection. The procedure was done according to manufacturer's guidelines as follows:

1- 500 µl from miniprep culture was inoculated into 100 ml LB-medium containing 50 µg/ µl, and grown at 37 ºC for 14 hours with vigorous shaking (~ 250 rpm).

2- The cells were divided into two 50 ml tubes and harvested by centrifugation at 4500 X g for 20 min.

3- Each bacterial pellet was resuspended in 10 ml buffer P1 (Resuspension buffer)

Buffer P1: 50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A.

4- 10 ml buffer P2 (lysis buffer) was added to each tube, mixed gently by inverting 4 - 6 times. Buffer P2: 200 mM NaOH, 1% SDS (w/v).

5- 10 ml buffer P3 (neutralization buffer) was added to each tube. The contents were mixed by gently inverting 4-6 times and put on ice for 15 min. Buffer P3: 3.0 M potassium acetate (PH 5.0).

6- After centrifugation at ≥ 20,000 g for 30 min at 4 ºC, the supernatant containing plasmid DNA was removed promptly.

7- The supernatant was centrifuged again at ≥ 20,000 g for 15 min (4ºC) and the supernatant containing plasmid DNA was removed promptly.

8- A QIAGEN-tip 100 was equilibrated by applying 4 ml Buffer QBT (equilibration buffer), and the column was allowed to empty by gravity flow. Buffer QBT: 750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v).

9- The two supernatants from step 7 were pooled and the sample was applied to the two QIAEN-tip and allowed to enter the resin by gravity flow.

10- The QIAGEN-tip was washed with 2x10 ml buffer QC (wash buffer). Buffer QC: 1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol (v/v).

11- DNA was eluted with 5x1 ml Pre-warmed to 65 4 ºC buffer QF (elution buffer). Buffer QF: 1.25 M NaCl, 50 mM Tris·Cl, pH 8.5, 15% isopropanol (v/v).

12- DNA was precipitated by adding 3.5 ml room-temperature isopropanol, mixed and centrifuged immediately at ≥ 15,000 x g for 30 min at 4 ºC. The supernatant was decanted carefully.

13- The DNA pellet was washed with 2 ml of room-temperature 70 % ethanol and centrifuged at ≥ 15,000 x g for 10 min. The supernatant was carefully decanted without disturbing the pellet.

14- After air-drying the pellet for 5-10 min, the DNA was dissolved in a 20 µl distilled water and the concentration was determined.

5.1.9. HEK 293 Cell Transfection and cell culture

HEK 293 cell line (Takara Bio Europe, Saint-Germain-en-Laye, France) was used for transfection and protein expression. HEK 293 cells were generated by transformation of human embryonic kidney cell cultures (hence HEK) with sheared adenovirus 5 DNA, and were first described in 1977.

Thawing of the cells

Cells were stored in liquid nitrogen, RPMI medium was added to dilute the toxic freezing medium dimethylsulfoxid (DMSO) and the cells were thawed in a water bath at 37ºC, then centrifuged at 3000 x g for 5 min. After that, the supernatant was disposed and the cells were resuspensed in a 750 ml flask filled with 500 ml medium and incubated at 37º C with 5% CO2. The cover of the culture bottles was not tightly closed to allow CO2 and humidity to enter the flask. Maintenance of the cells was performed twice a week with growth and maintenance medium (1:10) and then incubated at 37ºC with 5% CO2.

Cell transfection

Transfection of HEK 293 cells with pSfi-Express-HA vector containing MPHOSPH1 insert was done using FuGENE HD transfection reagent (Roche Applied science, Mannheim, Germany), the transfection was done following the manufacturer's guidelines as follows:

The plasmid DNA solution was prepared in sterile water at a concentration of 0.1 µg/ µl. For initial optimization experiments, a monolayer of cells that is 80 – 90% confluent was transfected in a six-well culture dish, using 3:2, 4:2, 5:2, 6:2, 7:2, and 8:2 ratios of FuGENE HD Transfection Reagent (µl) to DNA(µg), respectively. FuGENE HD Transfection Reagent, DNA, and diluent were allowed to adjust to +15 to +25°C. 100 µl diluents, containing 2 µg DNA, was Placed into each of six sterile tubes labeled 3:2, 4:2, 5:2, 6:2, 7:2, and 8:2. The FuGENE HD Transfection Reagents (3, 4, 5, 6, 7, or 8 µl) were directly transferred into the medium containing the diluted DNA without allowing contact with the walls of the plastic tubes. The tubes were vortexed for one to two seconds to mix the contents and the mixture was incubated for 15 minutes at room temperature. The transfection complex was added to the cells in a drop-wise manner. The wells were swirled to ensure distribution over the entire plate surface. After transfection, the cells were incubated for 18 – 72 hours prior to measuring the protein expression. After this incubation period, the protein expression was measured

using western blot analysis. Western blot analysis was applied to confirm size and specificity of the protein using a mouse anti-His monoclonal antibody (1:3000; Sigma, St Louis, MO) and a MPHOSPH1-specific mouse polyclonal antibody (1: 500; Abnova, Taiwan).

5.1.10. Western blot analysis

Purified proteins were prepared in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein were analyzed by SDS polyacrylamide gel electrophoresis (PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA), and incubated with blocking buffer overnight. Immunoblots were performed at 1:500 dilution of patients' serum, mouse anti-His monoclonal antibody or MPHOSPH1-specific mouse polyclonal antibody, respectively. Immunodetection was performed by incubation with horseradish peroxidase-conjugated antihuman IgG (1:10.000) or anti-mouse IgG secondary antibody (1:5000) as indicated by the host origin of the primary antibody and developed by chemiluminescence (NEN Life Science Products, Boston, MA).

5.2. Generation of desmoplakin expression construct

5.2.1. Amplification of desmoplakin construct with PCR

Amplification of desmoplakin cDNA was done using the following primers, as forward primer (5'-GGCTTCGAGGGTGTGAAGGGAAAGAA-3') and reverse primer (5'-TGCGGTGTCCCTTAAGAAGGATGA -3') with annealing temperature of 60 ºC.

Both primers were diluted with appropriate volume of distilled water to get the concentration of 100 pmol/µl, mixed 1:1 and stored at -20ºC until use. Before use, this stock solution was diluted 1:10 with distilled water.

The following PCR reaction components were applied: 2 µl Primers mix, 0.2 µl DNTPs, 3 µl Buffer Taq 10x, 0.2 µl Taq polymerase, 24.5 µl H2O and 0.5 µl DNA.

As negative control, 0.5 µl water instead of DNA was used, the amplification was performed using a PCR-program described in table 7:

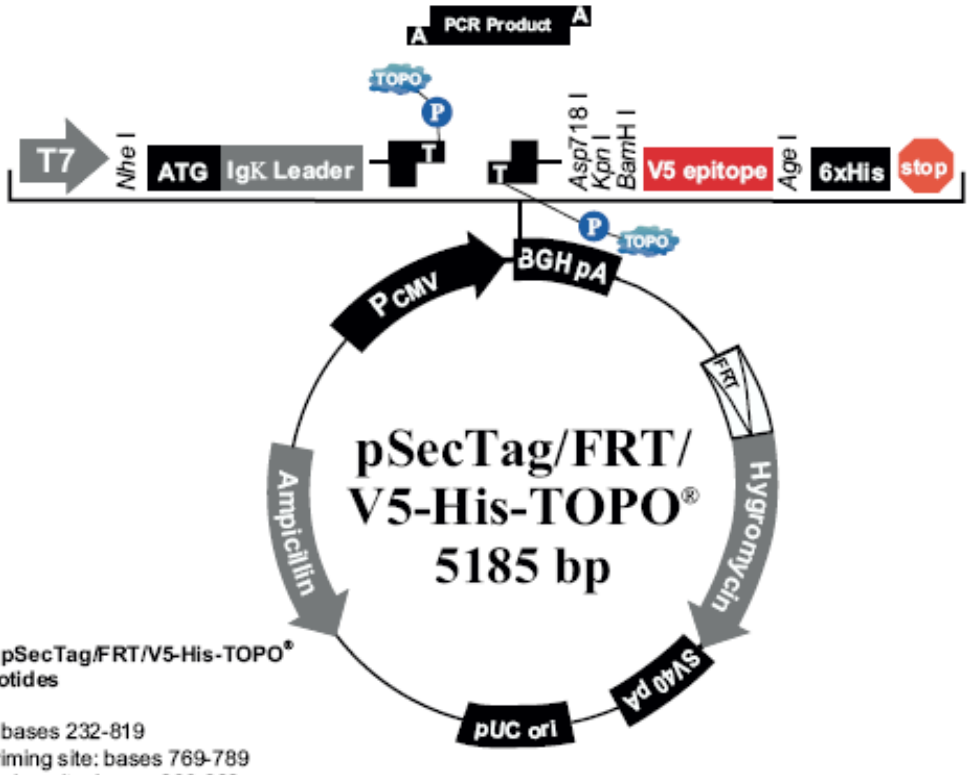
Table 7: PCR-program used for amplification of desmoplakin construct.

	Time (min: sec)	Temperature (ºC)	Cycle number
Hot start	12:00	94	1
Denaturation	01:00	94	35
Annealing	02:00	60	
Extension	01:00	72	
Final extension	9:00	72	1

After amplification, the DNA product was mixed with blue marker. Using 1 % agarose gel, the DNA was separated according to the molecular weight for 45 minutes. The next step was to detect the products using ultra violet light source and by comparing with DNA-marker VIII, the position of desmoplakin band could be determined (~ 2 Kbp).

5.2.2. DNA cloning with pSectag/FRT/V5-His topo TA Expression vector

pSectag/ FRT/V5-His topo TA Expression vector (Invitrogen- Germany) was used for the cloning (Figures 21 and 22). In an Eppendorf 0.5 µl PCR product was added to 1 µl salt solution, 0.5 µl Topo vector and 3 µl distilled Water. Mixed the reaction gently and incubated for 5 minutes at room temperature (22-23°C). The mixture was placed on ice and continued to transforming competent cells. The product was tested with gel electrophoresis to determine the desmoplakin Band (~ 2000 bp).



Comments for pSecTag/FRT/V5-His-TOPO®
5185 nucleotides

CMV promoter: bases 232-819
CMV forward priming site: bases 769-789
T7 promoter/priming site: bases 863-882
IgK secretion signal: bases 905-967
TOPO® Cloning site: bases 1012-1013
V5 epitope: bases 1046-1087
Polyhistidine (6xHis) region: bases 1097-1114
BGH reverse priming site: bases 1137-1154
BGH polyadenylation signal: bases 1143-1367
FRT site: bases 1651-1698
Hygromycin resistance gene (no ATG): bases 1706-2725
SV40 early polyadenylation signal: bases 2858-2988
pUC origin: bases 3371-4044 (complementary strand)
bla promoter: bases 5050-5148 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4189-5049 (complementary strand)

Figure 21: Schematic presentation of pSecTag/FRT/V5-His-TOPO vector (5185 bp).

Figure 22: Sequence of the cloning sites of pSectag/FRT/V5-His topo vector.

5.2.3. Cell transfection with the vector

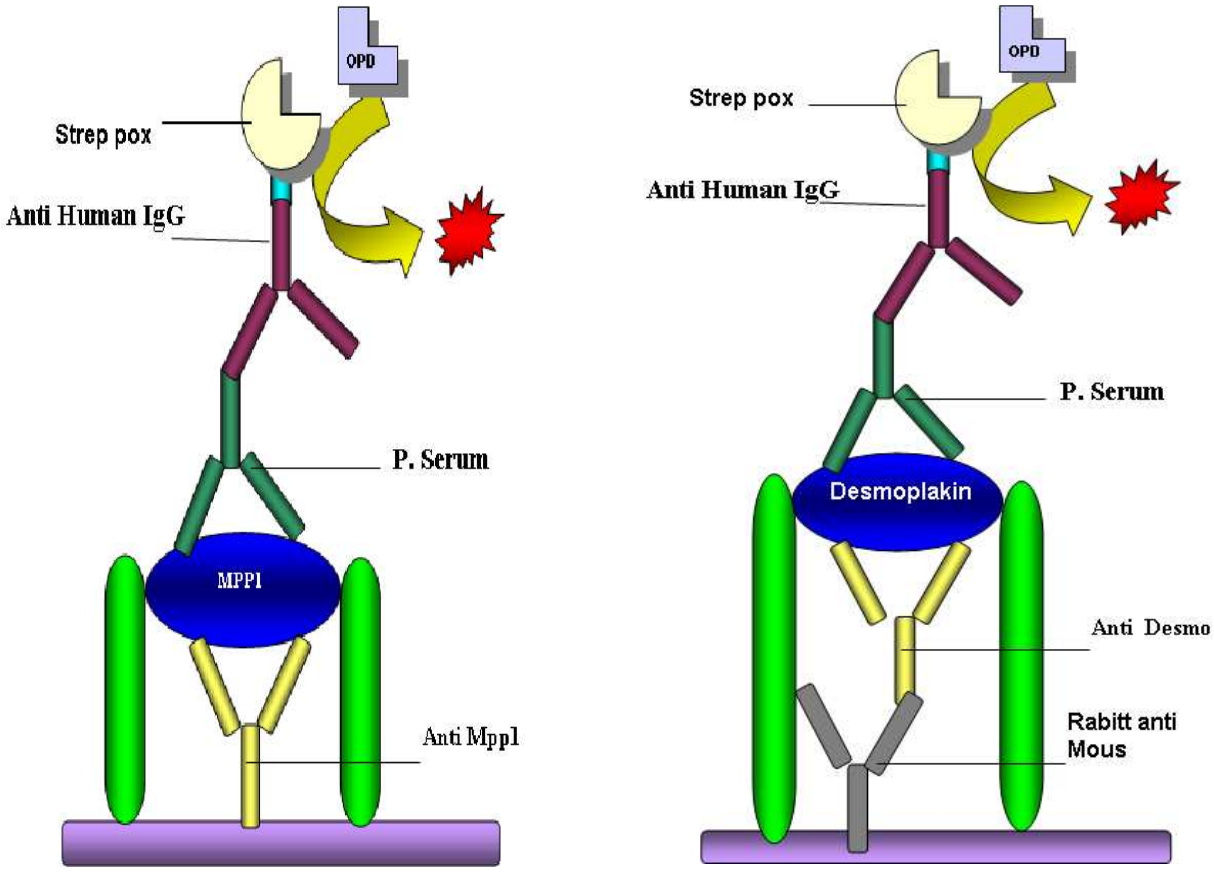
On the day of transfection, HEK 293 cells were tested; the cell confluency has to be about 70-80 %. For Transfection, FuGENE HD Transfection (Roche Applied science - Mannheim, Germany) was used. The transfection complex was done according to manufacturer's instructions as follows:

Five µg DNA was suspended in 100 µl RPMI medium, for transfection control, 5 µg green fluorescent protein (GFP) instead of DNA was used. After that, 7.5 µl FuGENE were added carefully according to transfection ratio (DNA/FuGENE = 2/3), mixed gently and stored 15 minutes at room temperature. The transfection mixture was then added to the cells growing in 2 mL RPMI Medium 1640 medium (Invitrogen- Germany) with 10 % v/v fetal calf serum (FCS) and was grown at 37ºC with 5% CO2. After three days, the growing of the cells was tested and 1 mg/ml Geneticin G 418 sulfate (which is an aminoglycoside antibiotic similar in structure to gentamicin B1) was added.

Western blot analysis was applied using a mouse anti-His monoclonal antibody (1:3000; Sigma, St Louis, MO, USA) and desmoplakin 1&2 mouse monoclonal antibody (1:2000, Biotechnica, Germany) as described by MPHOSPH1.

5.3. Titration of antibodies by ELISA

In order to determine the titers of antibodies against the two gene products in the sera of PNH patients and healthy controls; sera were tested for reactivity against desmoplakin and MPHOSPH1 by ELISA. Plates were coated with mouse anti MPHOSPH1 antibody for MPHOSPH1 and rabbit anti mouse antibody followed by mouse anti-desmoplakin antibody for desmoplakin (Figure 23).



Elisa for MPHOSPH 1 Elisa for Desmoplakin

Figure 23: ELISA system for desmoplakin and MPHOSPH1.

Plates were washed with PBS and blocked overnight at + 4°C with 5% nonfat dry milk in TBST. After washing the plates with PBS, 100 µl 1:1 cell lysate were added to each well and incubated at 37º C for two hours. Using PBS, the plates were washed three times and patients' sera were added to a final dilution of 1:1000 and incubated at room temperature for 2 hours.

After washing, the plates were incubated with alkaline phosphatase-conjugated goat antihuman IgG antibody (1:7500; Promega) at room temperature. Finally, the plates were washed and incubated with *p*-nitrophenyl phosphate (PNPP) substrate (Pierce, Rockford, IL) at room temperature, and the optic density (OD) at 405 nm was determined. A positive reaction was defined as an absorbance value exceeding the mean OD absorbance value of sera from healthy donors by three standard deviations.

6. Epitope mapping

In order to detect hot spots of epitopes recognized by anti-desmoplakin antibodies and anti-MPHOSPH1 of different patients, epitope mapping was performed using PCR with different primers that amplify small fragments (~ 200 bp) . After amplification, fragments were ligated individually into pSectag/FRT/V5-His topo TA expression vector (Invitrogen- Germany). Using FuGENE HD Transfection (Roche Applied science - Mannheim, Germany); HEK293 cells were transformed with each of the constructed pSectag/FRT/V5-His topo TA Vector for peptide expression. An ELISA system was established by coating wells with anti-HIS antibodies followed by cell lysates from each of the culture mentioned above. After washing, patients' sera, that showed reactivity against desmoplakin and MPHOSPH1, were added. After that, plates were incubated with alkaline phosphatase-conjugated goat antihuman IgG antibody (1:7500; Promega) at room temperature. Finally, the plates were washed and incubated with p-nitrophenyl phosphate (PNPP) substrate (Pierce, Rockford, IL) at room temperature, and the optic density (OD) at 405 nm was determined.

7. Construction of cDNA Library from $CD34^+$ Cells

7.1. $CD34^+$ cells Isolation

400 ml peripheral blood with EDTA was used from normal individual to isolate $CD34^+$ cells. The selection was done using CD34 MicroBead Kit (Miltenyi Biotec, Germany). The following steps were done according to the manufacturer's instructions:

7.1.1. Isolation of peripheral blood mononuclear cells (PBMCs) using Ficoll-paque

The cells were diluted with four volumes of phosphate buffer saline (PH 7.2) supplemented with 2 mM EDTA. Over 15 ml Ficoll-Paque (GE Healthcare companies, Germany) 35 mL of diluted cell suspension was layer carefully in a 50 mL conical tube and centrifuged at 400xg for 40 minutes at 20 °C in a swinging bucket rotor without brake. After that, the upper layer

was aspirated leaving the mononuclear cell layer undisturbed at the interphase. The interphase contains lymphocytes, monocytes and thrombocytes. The interphase was transferred into a new 50 mL conical tube. The tube was filled with PBS containing 2 mM EDTA, mixed and centrifuged at 300 xg for 10 minutes at +20°C and the supernatant was removed completely. In order to remove the platelets, the cell pellet was resuspended in 50 mL of buffer, centrifuged at 200 xg for 15 minutes at +20 °C and the supernatant was removed completely. In order to remove the platelets completely, the last step was repeated. The cell pellet was resuspended in a final volume of 300 µL of buffer and stored in +4 °C for use.

7.1.2. Magnetic labeling and separation

The magnetic labeling and separation was made using the protocol mentioned above (see 4.1.2 and 4.1.3). After separation, cells were counted using a Neubauer counting chamber. The purity of $CD34^+$ cells was tested using flow cytometry.

7.2. Extraction of mRNA

7.2.1. Extraction of total RNA

RNeasy Mini kit (Qiagen, Germany) was used to extract total RNA from the selected cells. Before starting, the reagents provided by the kit were prepared:

10 µl β-mercaptoethanol (β-ME) was added to 1 ml RLT buffer. The buffer RPE was supplied as a concentrate, so that 4 volumes of 100% ethanol were added to make the working solution. RNA extraction was done according to the manufacture's guidelines as follows:

1. The cell suspension was centrifuged for 5 min at 300 xg and the supernatant was removed carefully, the cells were disrupted by flicking the tube and adding 350 µl buffer RLT, mixed well by vortexing and proceeded to the next step.

2. The lysate was pipeted into a QIAshredder spin column placed into a 2 ml collection tube, centrifuged for 2 min at full speed. After that, one volume of 70 % ethanol was added to the homogenized lysate and mixed well by pipetting.

3. Up to 700 µl of the sample was transferred to an RNeasy spin column placed into a 2 ml collection tube, the lid was closed, centrifuged for 15 seconds at ≥ 8000 g (≥10.000 rpm) and the flow-through was discarded.

4. 700 µl buffer RW1 was added to the RNeasy spin column, the lid was closed, centrifuged for 15 seconds at ≥ 8000 g (≥10.000 rpm) to wash the spin column membrane, and the flow-through was discarded.

5. 500 µl buffer RPE was added to the column, the lid was closed, centrifuged for 15 seconds at ≥ 8000 g (≥10.000 rpm) and the flow-through was discarded. 500 µl Buffer RPE was added again to the column, the lid was closed, centrifuged for 2 min at ≥ 8000 g (≥10.000 rpm) and the flow-through was discarded.

6. The RNeasy spin column was placed into a new 1.5 mL collection tube, 40 µl RNase-free water was added directly to the column membrane, the lid was closed and centrifuged for 1 min at ≥ 8000 g (≥10.000 rpm) to elute the RNA. Step 6 was repeated using another 40 µl RNase-free water in order to have the required amount, which was 90 µg total RNA.

7.2.2. Purification of mRNA

For the purification of mRNA, Dynabeads Purification Kit (Invitrogen, Germany) was used. The isolation of mRNA relies on base pairing between the poly A residues at the 3' end of most mRNA and the oligo $(dT)_{25}$ residues covalently coupled to the surface the surface of the Dynabeads. Other RNA species lacking a poly A tail will not hybridize to the beads and are readily washed away. The following buffers were provided: Binding Buffer (20 mM Tris-HCl {pH 7.5}, 1.0 M LiCl, 2 mM EDTA), Washing Buffer B (10 mM Tris-HCl {pH 7.5}, 0.15 M LiCl, 1 mM EDTA), and 10 mM Tris-HCl (pH 7.5).

Purification of mRNA was done according to the following steps:

1. The volume of total RNA to100 µl was adjusted with 10 mM Tris-HCl, heated to 65°C for 2 minutes to disrupt the secondary structures and it was finally placed on ice.

2. As a next step, 200 µl of resuspended Dynabeads were transferred from the stock tube suspension, dispensed into a 1.5 ml microcentrifuge tube, and placed on the magnet. After 30 seconds, the supernatant was pipetted off, the tube was removed from the magnet, the Dynabeads were resuspended in 100 µl binding buffer, and the tube was placed back on the magnet. After 30 seconds, the supernatant was pipeted off and the tube was removed from the magnet.

3. 100 µL binding buffer was added to the Dynabeads. The total RNA was added to the 100 µL Dynabeads/Binding Buffer suspension, mixed thoroughly and rotated on a roller for 3–5 minutes at room temperature to allow mRNA to anneal to the oligo $(dT)_{25}$ on the beads.

4. The tube was placed on the magnet until solution became clear, the supernatant was removed, the tube was removed from the magnet, and the mRNA-bead complex were washed twice with 200 µl Washing Buffer B. The mRNA was eluted using 15 µL mM tris-HCl and the eluted mRNA was transferred to a new RNase-free Tube.

7.3. First-Strand cDNA synthesis

For this step, a switching mechanism at 5' end of RNA transcript (SMART) cDNA Library Construction Kit (Takara Bio Europe, Saint-Germain-en-Laye, France) was used. The following components were necessary:

- SMART IV Oligonucleotide (12 µM)

  5´-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3`

- CDS III/ 3`PCR primer (12 µM)

  5`-ATTCTAGAGGCCGAGGCGCCGACATG-d(T)30N_1N-3`

  (N=A, G, C, or T; N_1= A, G, or C)

- SMARTScribe MMLV Reverse Transcriptase (100 units /µL)
- 5X First-Strand Buffer, which consist of the following ingredients:

  250 mM Tris (pH 8.3)

  30 mM MgCl2

  375 mM KCL

- DTT (dithiotreitol;20 mM)
- Control poly A^+ RNA (Human Placenta; 1.0 µg/ µL)
- DNA size markers
- 1.1% Agarose/EtBr gel (containing 0.1 µg/mL ethidium bromide)

The CDS III/ 3`PCR primer is a modified oligo (dT) primer, which primes the first-strand synthesis reaction, and the SMART IV Oligo serves as a short extended template at the 5' end of the mRNA. When the reverse transcriptase (RT) reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end

of the cDNA. The SMART IV Oligo, which has an oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT switches the templates and continues replacing to the end of the Oligonucleotide. The resulting full-length single strand cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo, which then serves as a universal priming site (SMART anchor) in the subsequent amplification by long distance PCR. According to the manufacture's guidelines the following steps were done:

In a sterile 0.5-mL microcentrifuge tube, the following reagents were combined: 1 µL RNA sample (or 1 µL control RNA for the control) + 1 µL SMART IV Oligonucleotide + 1 µL CDS III/3' PCR primer. A total volume up to 5 µL was obtained using deionized H_2O. The contents were mixed and the tube spun briefly in a microcentrifuge. The tube was incubated at 72 °C for 2min. The tube was cooled on ice for 2 minutes; the contents were collected at the bottom of the tube by briefly spun. The following contents were added to the tube:

2.0 µL First Strand Buffer

1.0 µL DTT (20 mM)

1.0 µL dNTP Mix (10mM)

1.0 µL SMARTScribe MMLV Reverse Transcriptase

The contents were mixed by gently pipetting and were briefly centrifuged. The tube was incubated at 42 °C for 1 hour using a hot lid thermal cycler. After the incubation, the tube was placed on ice in order to terminate first-Strand synthesis. 2 µL from the mixture for PCR step was put in a clean 0.5-mL tube, and the tube was placed on ice. The rest of mixture was stored at -20 °C.

7.4. Amplification of cDNA by PCR

For the amplification is a 5' PCR Primer necessary, the primer has the following sequence:

5'-AAGCAGTGGTATCAACGCAGAGT-3'

The Advantage 2 Polymerase Mix is used, which is a mix of TITANIUM Taq DNA polymerase (Takara Bio Europe, Saint-Germain-en-Laye, France) - a nuclease- deficient N-terminal deletion of Taq DNA polymerase plus Start Antibody to provide automatic hot-start PCR- and a minor amount of proof reading polymerase. This system allows to amplify

efficiently full-length cDNA with a fidelity rate significantly higher than that of conventional PCR.

The PCR thermal cycler was preheated to 95 °C and the following components were added to the reaction tube:

2 µL first-strand cDNA

80 µL deionized H2O

10 µL 10x PCR buffer

2 µL 50x dNTP mix

2 µL 5' PCR primer

2 µL CDS III/3' PCR primer

2 µL 50x Advantage 2 Polymerase Mix

The contents were mixed by flicking the tube gently and the contents were collected at the bottom of the tube by briefly centrifugation. The tube was placed in a preheated thermal cycler. The amplification was done at annealing temperature of 68 °C for 6 min and 25 PCR cycles.

After the completion of the cycling, 5 µL of the PCR product was analyzed alongside 0.1 µg of 1-kb DNA size markers, on a 1.1 % agarose/EtBr gel.

7.5. Proteinase K digestion

Digestion proteinase K (20 µg/µL) was used for this step. In a sterile 0.5-mL tube, 50 µl of amplified ds cDNA and 2 µL of proteinase K were mixed, and the tube was spun briefly. The mixture was incubated at 45 °C for 20 min, the tube spun briefly. 50 µl of deionized H_2O and 100 µL of phenol:chloroform:isoamyl alcohol were added to the tube and mixed by continuous gentle inversion for 2 minutes. The phases were separated by centrifugation at 14.000 rpm for 5 min. The top layer was moved to a clean 0.5-ml tube and the rest was discarded. To the aqueous layer, the following solutions were added: 10 µl of 3 m sodium acetate, 1.3 µl of glycogen (20 µg/µL) and 260 µL of room temperature 95% ethanol. Then, it was centrifuged at 14.000 rpm for 5 min and the phases were separated. The supernatant was removed, without disturbing the pellet, using a pipette. After that, the pellet was washed with 100 µl of 80% ethanol and air dried for 10 min. 79 µL of deionized H_2O was added to

resuspend the pellet.

7.6. Sfi L digestion

Sfi L enzyme was used for the digestion because its asymmetrical restriction sites at the 5' and 3' cDNA ends, the recognition sequences of both SfilA and SfilB is shown in table 8:

Table 8: The recognition sites of SfilA and SfilB enzymes.

Sfi L A site	**Sfi L B site**
5'-GGCC**ATTAC**GGCC-3' 3'-CCGG**TAATG**CCGG-5'	5' – GGCC**GCCTC**GGCC – 3' 3' – CCGG**CGGAG**CCGG – 5'

For this step, the following components were used: Sfi LEnzyme (20 units/µL), 10x Sfi L buffer, 100x BSA.

In a fresh 0.5-ml tube, 79 µL cDNA, 10 µL 10x Sfi Buffer, 10 µL Sfi L enzyme and 1 µL 100x BSA were combined, mixed well and incubated at 50 °C for 2 hours. Then 2 µL of 1 % xylene cyanol dye was added to the tube and mixed well.

7.7. Size fractionation of cDNA by chroma SPIN-400

Sixteen 1.5-ml tubes were labeled and arranged in a rack in order, then the CHROM Spin-400 column (which is a spin column packed with gel filtration resin to rapidly purify and size select nucleic acid samples) were prepared for drop procedure. The column was inverted several times to resuspend the gel matrix completely. The air bubbles were removed from the column. The column was attached to a ring stand, and the storage buffer was drained through it by gravity flow, until the surface of the gel beads in the column matrix became visible. The top of the column matrix should be at 1.0-ml mark on the wall of the column and the flow rate should be approximately 1 drop/40-60 sec. After the storage buffer stopped dripping; 700 µL buffer was added to the top of the column and allow it to drain out. When this buffer stopped dripping (~ 15-20 min), 100 µl mixture of Sfil-digested cDNA and xylene cyanol dye was applied to the top-centre surface of the matrix. The sample was left to be absorbed by the surface of the matrix.

The tube, which contained the cDNA, was washed with 100 µL of column buffer and applied to the surface of the matrix. After the buffer dripped out the column, the rack containing the collection tubes was placed under the column, 600 µL of column buffer was added to the column, and single-drop fractions were immediately collected. In order to test the fractions, electrophoresis (at 150 V for 10 min) of 3 µl of each fraction on a 1.1 % agarose/EtBr gel alongside 0.1 µg of 1-kb DNA size marker should be made. The peak fractions were determined by visualizing the intensity of the bands under UV. The first three fractions containing cDNA were collected and pooled in a clean 1.5-ml tube. The following reagents were added to the tube containing cDNA fractions:

1/10 vol Sodium Acetate (3M; pH 4.8)

1.3 µl Glycogen (920 mg/ml)

2.5 vol 95% ethanol (-20 °C)

After mixing gently, the tube was placed in -20 °c for 1 hour, and then centrifuged at 14.000 rpm for 20 min at room temperature. The supernatant was removed with a pipette without disturbing the pellet, the process was repeated, and the pellet was air dried for 10 min. The pellet was resuspended in 7 µL of deionized water and mixed gently. The Sfil-digested cDNA was ready to be ligated to the Sfil- digesed, diphosphorylated λ TripelEx2 vector.

7.8. Ligation of cDNA to λ TriplEx2 vector

The following protocol is optimized for ligation of SMART cDNA to the λ TriplEx2 DNA: A test ligation was made to determine the efficacy of ligating the vector to the control insert. 1 µL of vector, 1 µL of control insert, 1.5 µL of deionized H_2O, 0.5 µL 10xligation buffer, 0.5 µL ATP (10 mM) and 0.5 µL T4 DNA Ligase were used. Ligation mixture was incubated at 16 °C overnight. After that, a λ-phage packaging reaction was applied and the titers of the resulting phages were determined, the ligation was considered efficiency if $\geq 1x10^7$ pfu/µg of input vector were obtained. In each of three 0.5-ml tubes, 1 µL of vector, 0.5 µL of cDNA, 2 µL of deionized H_2O, 0.5 µL 10xligation buffer, 0.5µL ATP (10 mM) and 0.5 µL T4 DNA ligase. The tubes were mixed gently without producing air bubbles, briefly centrifuged to bring contents to the bottom of the tubes. The tubes were incubated at 16 °C overnight. After that, a λ-phage packaging reaction was performed and the titer of the resulting phages for each of the ligations was determined. The unamplified libraries can be stored at 4 °C for 2

weeks.

7.9. Bacterial culture plating

First, the frozen cells of (E.coli XL1-Blue and BM.25) were recovered by streaking small portion (5µL) of the frozen stock onto an LB agar plate containing the appropriate antibiotic (LB agar with tetracycline for XL1-Blue stock plates and kanamycin) this was the primary streak plate. The LB agar was incubated at 37 °C overnight. After that, it was covered with parafilm and stored at 4 °C. A single isolated colony from the primary streak plate was picked and streaked onto another LB/$MgSO_4$ agar plate without antibodies. The plate was incubated at 37 °C overnight. The plate was covered with parafilm and stored at 4 °C for two weeks. This plate is used as a source of fresh colonies for inoculating liquid cultures and preparing the next fresh working stock plate. At 2-weeks intervals, a fresh working stock plate was prepared from the previous working stock plate.

7.10. Titration of unamplified library

Determining the titer of the unamplified library gives an estimate of the number of independent phage and independent clones in the library, which helps to determine the efficiency of the ligation of vector to positive control insert and the background titer of the vector alone. The titration was made according to the following steps:

1. A single, isolated colony from the working stock plate was picked and used to inoculate 15 ml LB/$MgSO_4$/ maltose broth in a 50-ml test tube or erlenmeyer flask. Then it was incubated at 37 °C overnight while shaking (at 140 pm) until the OD600 of the culture reached (2.0).The cells were centrifuged at 5.000 rpm for 5 min, the supernatant was poured off, and the pellet was resuspended in 7.5 ml of 10 mM $MgSO_4$.

2. A number of 90-mm LB/$MgSO_4$ plates were warmed and dried.

3. Dilutions (1:5, 1:10, 1:15, and 1:20) of each of the packaging extracts (from 7.8) were made and the following steps for each dilutions of each extract were performed:

1 µl of the diluted phage was added to 200 µL of the XL1-blue overnight culture and allowed the phage to adsorb at 37 °C for 10-15 min. 2 ml of melted LB/$MgSO_4$ top agar was also added, mixed by quickly inverting and immediately poured onto 90-mm LB/$MgSO_4$ plates prewarmed to 37 °C. The plates were swirled quickly after pouring to allow even distribution

of the top agar. The plates were cooled at room temperature for 10 min to allow the top agar to harden, inverted and incubated them at 37 °C for 16 hours. The number of the growing plaques was counted and the titer of the phage (pfu/ml) was calculated according to the following equation:

$$\text{Pfu/ml} = \frac{\text{number of plaques x dilution factor x } 10^3\ \mu\text{L/ml}}{\mu\text{L of diluted phage plated}}$$

By 1:1 vector: insert, $1x10^7$ clones were obtained, which means that the ratio was optimal for the ligation.

7.11. Determination of the percentage of recombinant clones

To perform blue/white screening in E.coli XL1-Blue, the same procedure of titration an unamplified library was followed (Paragraph 7.10) except adding IPTG and X-gal to the melted top agar before plating the phage + bacteria mixtures. For every 2 ml of melted top agar, 50 µL of the IPTG and X-gal stock solutions were used. The plates were then incubated at 37 °C until plaques and blue color developed (~14 hours). The ratio of white (recombinant) to blue (non-recombinant) plaques gives a quick estimate of recombination efficiency.

7.12. Library amplification

1. A single, isolated colony from the primary working plate of XL1-Blue was picked and used to inoculate 15 ml LB/$MgSO_4$/ maltose broth. The culture was incubated at 37 °C overnight while shaking (at 140 pm) until the OD600 of the culture reached (2.0). The cells were centrifuged at 5.000 rpm for 5 min, the supernatant was poured of, and the pellet was resuspended in 7.5 ml of 10 mM $MgSO_4$.

2. A number of 90-mm LB/$MgSO_4$ plates were warmed and dried.

3. The required number of 4-ml tubes with 500 µL of overnight bacterial culture and enough diluted lysate were added to have $6\text{-}7x10^4$. The mixture was incubated in a 37 °C water bath for 15 min. After that, 4.5 ml of melted LB/$MgSO_4$ soft-top agar was added to each tube.

4. The mixture of bacteria and phages was poured onto LB/$MgSO_4$ agar plates.

5. The plates were swirled quickly after pouring to allow even distribution of the top agar.

The plates were cooled at room temperature for 10 min to allow the top agar to harden, then they were inverted and incubated at 37 °C for 16 hours.

6. To each plate, 12 ml of 1xlambda dilution buffer was added, stored at 4 °C overnight. The plates were put on platform shaker (~ 50 rpm), the plates were incubated at room temperature for 1 hour. The λ-phage lysate was poured into a sterile beaker.

7. To clear the phage lysate of cell debris and to lyse any remaining intact cells, the phage lysate was mixed well and poured into a sterile, 50-ml polypropylene, screw-cap tube. 10 ml of chloroform was added to the lysate, screw on the cap and vortexed for 2 min, centrifuged at 7.00 rpm, and collected the supernatant into another sterile 50-ml tube. The cap was tightly closed and place at 4 °C. The amplified library can be stored at 4 °C for up to 6 months. For long-term storage; 1-ml aliquots were made, DMSO was added to a final concentration of 7 % and placed at -70 °C.

7.13. Titration of the amplified library

The same procedure that used in the titration of an unamplified library was used as follows:

1. A single, isolated colony from the primary working plate of XL1-Blue was picked and used to inoculate 20 ml LB/$MgSO_4$/ maltose broth (without antibiotic). After that, it was incubated at 37 °C overnight while shaking (at 140 rpm) until the OD_{600} of the culture reached (2.0). The cells suspension was centrifuged at 5.000 rpm for 5 min, poured of the supernatant, and resuspended the pellet in 7.5 ml of 10 mM $MgSO_4$. Warm and dry four LB/$MgSO_4$ agar plate (90-mm size) as explained above.

2. The following dilutions of the lysate (library) were prepared: Onto 1x lambda dilution buffer, 10 µl of the library lysate (dilution 1= 1:100) was pipeted, 10 µl of dilution 1 was transferred into a second tube containing 1mL of 1x lambda dilution buffer (Dilution 2 = 1:10.000). Using XL1-Blue overnight culture, obtained from the last step, and phage dilution 2, four tubes were prepared. Table 9 exhibits the contents of the four tubes. The tubes were incubated in 37 °C water bath for 15 min, 3 ml of melted (45 °C) LB/$MgSO_4$ top agar were added to each of the four tubes, mixed well and poured the contents from each tube onto separate LB/$MgSO_4$ agar plates (The plates were swirled quickly as mentioned above).

3. The plates were cooled at room temperature for 10 min, incubated at 37 °C (inverted

position) for at least 7 hours. After the growing of the plaques, they were counted and the titer was calculated according to the equation in (Paragraph 7.10). A titer of 2x 10^{10} pfu/ml was obtained. The library was stored in 4 °C and was ready for the screening.

Table 9: Plating dilutions for titration of an amplified library.

1x Lmbda Tube	1x Lambda dilution buffer	Bacterial overnight culture	Phage Dilution2
1	100 µl	200 µl	5 µl
2	100 µl	200 µl	10 µl
3	100 µl	200 µl	20 µl
4 (Control)	100 µl	200 µl	0 µl

8. Immunoscreening of cDNA expression library using MDS Sera

Sera from 10 MDS patients from Department of Hematology, Oncology and Clinical Immunology, Düsseldorf University Hospital (Düsseldorf, Germany) were used to screen human fetal liver cDNA library and cDNA library from normal $CD34^+$ cells, using the SEREX approach as described above (Paragraph 1). First, each serum was diluted at 1/10 with PBS, pooled and saved at 4°C. The serum pool was screened for antibodies against any products in both libraries. About 1x10^6 clones from each library were screened.

VI. Results

1. Screening with MDS sera

No reaction could be found by MDS sera neither by using fetal liver cDNA library nor by cDNA isolated from a normal individual $CD34^+$. Similarly, the MDS sera did not react with the gene products isolated with PNH sera.

2. Sequence analysis of defined antigens

The SEREX approach was applied to identify antibodies against auto-antigens using the sera of patients with PNH. By screening more than 1 x 10^6 phage plaques from a human fetal liver cDNA library with 1/100 diluted sera from 10 PNH patients, the products of two genes were identified (Figure 24). Lineage-specific expression was determined by searching the National Center for Biotechnology Information (NCBI) UniGene database (http://www.ncbi.nlm.nih.gov/UniGene/):

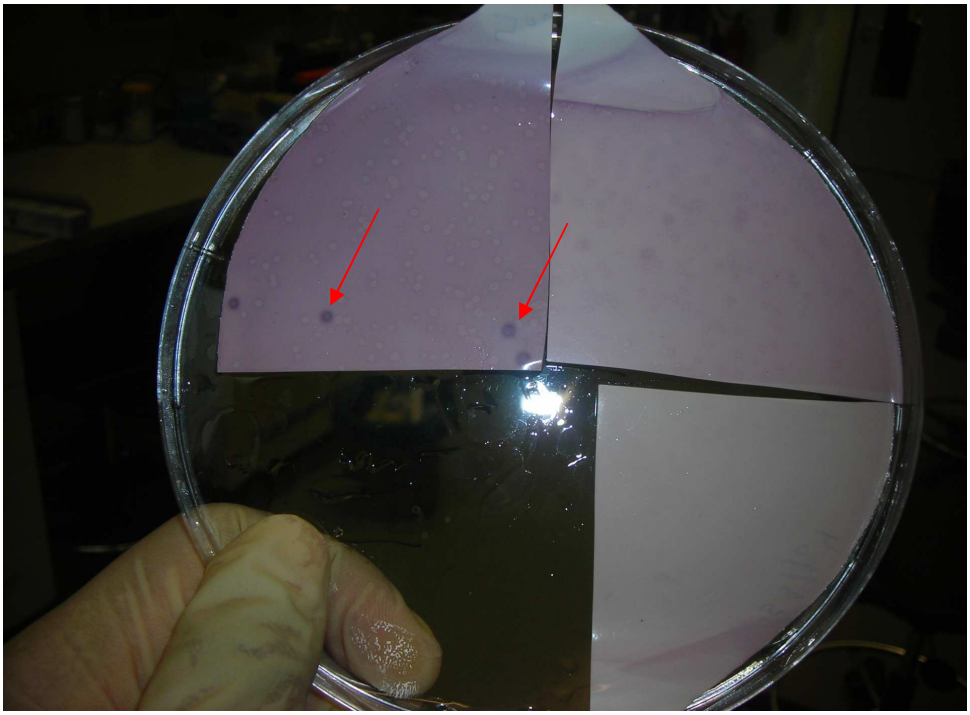


Figure 24: Positive signals using the SEREX approach.

2.1. MPHOSPH1

The first antigen was M-phase phosphoprotein 1(MPHOSPH1) (Termed also: kinesin family member 20B or KIF20B), which is a member of a set of mitotic phosphoproteins specifically recognized by the MPM2 antibody at the G_2/M transition. MPHOSPH1 is a 225-kd protein, previously known as a slow plus-end-directed kinesin-related protein (KRP) that plays critical roles in cytokinesis (MatsumotoTaniura *et al*, 1996b). It has a specific pattern of localization

and expression during the cell cycle, being mostly nuclear in interphase cells with a sharp increase in expression in G_2, and diffuse in metaphase cells, with subsequent association to the central spindle and the midbody at the end of mitosis. MPHOSPH 1 is a novel KRP whose activity is required for proper progression of cytokinesis in human cells. Moreover, using immunoblots analysis of MPHOSPH 1 distribution in various human tissues, it had been demonstrated that there is no expression of the protein by adult liver tissue (Abaza *et al*, 2003b).

2.2. Desmoplakin

The second antigen was encoded by the desmoplakin gene which encode a 240 kd protein associated with desmosomes. Desmosomes are intercellular junctions that tightly link adjacent cells. Desmoplakin is an obligate component of functional desmosomes that anchors intermediate filaments to desmosomal plaques (Figure 25). The N-terminus of desmoplakin is required for localization to the desmosome and interacts with the N-terminal region of plakophilin 1 and plakoglobin. The C-terminus of desmoplakin binds with intermediate filaments. In the mid-region of desmoplakin, a coiled-coiled rod domain is responsible for homodimerization. Mutations in this gene are the cause of several cardiomyopathies and keratodermas as well as the autoimmune disease paraneoplastic pemphigus (Jiao & Bystryn, 1998b). There is no sufficient information about the tissue expression of desmoplakin, but it is known that desmosomes are major sites of intercellular contact found in a variety of cells such as epithelial cells, cardiac myocytes, arachnoidal cells of meanings, and dendritic cells of germinal centers in lymph nodes (Virata *et al*, 1992).

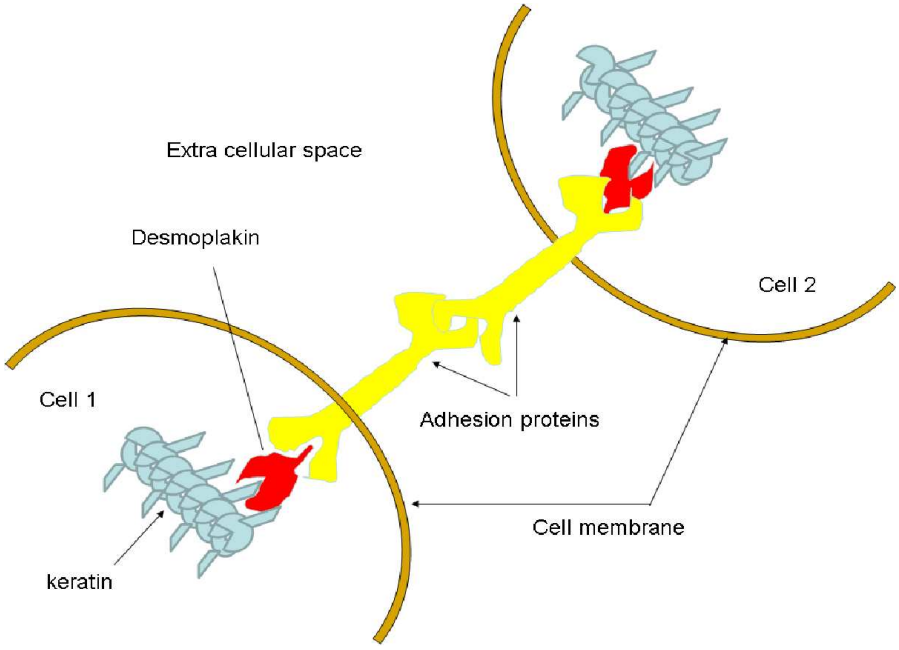


Figure 25: Cell adhesion in desmosomes. On the cytoplasmic side of the plasma membrane, there are two dense structures called the Outer Dense Plaque (ODP) and the Inner Dense Plaque (IDP). These are spanned by the desmoplakin protein. The Outer Dense Plaque is where the cytoplasmic domains of the cadherins attach to desmoplakin via plakoglobin and plakophillin. The Inner Dense Plaque is where desmoplakin attaches to the intermediate filaments of the cell.

3. Serological analysis of SEREX-defined antigens

The isolated clones were used to screen for antibodies in the sera of patients with PNH and the healthy donors. Three and four of the 10 PNH sera exhibit a positive reaction against MPHOSPH1 and desmoplakin, respectively.

In PNH sera 1, 5 and 9 a positive reaction against MPHOSPH1 was detected, and in PNH sera 5, 6, 7 and 10 against desmoplakin. Only in one healthy serum number 6 a positive reaction against desmoplakin was found. No reaction against either clone was found in any of aplastic anemia sera. The results of the screening were summarized in table 10.

Table 10: Summary of antibody screening.

	MPHOSPH 1	Desmoplakin
PNH sera (10)	3 (30%)	4 (40%)
Aplastic anemia (5)	No reaction	No reaction
Control sera (20)	No reaction	1 (5%)

Using PBS, dilution series were made for each of sera that exhibited reaction against desmoplakin and/ or MPHOSPH1 (1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$ and $1/10^6$) and the reaction against both of the clones was tested. Even with dilution factor $1/10^5$, the positive reactions against MPHOSPH1 were identified in PNH sera numbers 1, 5 and 9. In PNH sera 5, 6, 7 and 10 a positive reaction against desmoplakin were detected. The positive reaction in control serum number 6 against desmoplakin disappeared by diluting the serum at a factor $1/10^3$.

4. Detection of IgG antibodies against desmoplakin and MPHOSPH1 by ELISA and Western blot analysis

In order to determine the titers of antibodies against the two gene products in the sera of patients with PNH, aplastic anemia and healthy controls, sera were tested for reactivity against desmoplakin and MPHOSPH1 by ELISA. A serum was considered positive in the MPHOSPH1 ELISA if its absorbance value exceeded the mean absorbance value of sera from healthy donors by three standard deviations. The results of ELISA for MPHOSPH1 are depicted in (Figure 26).

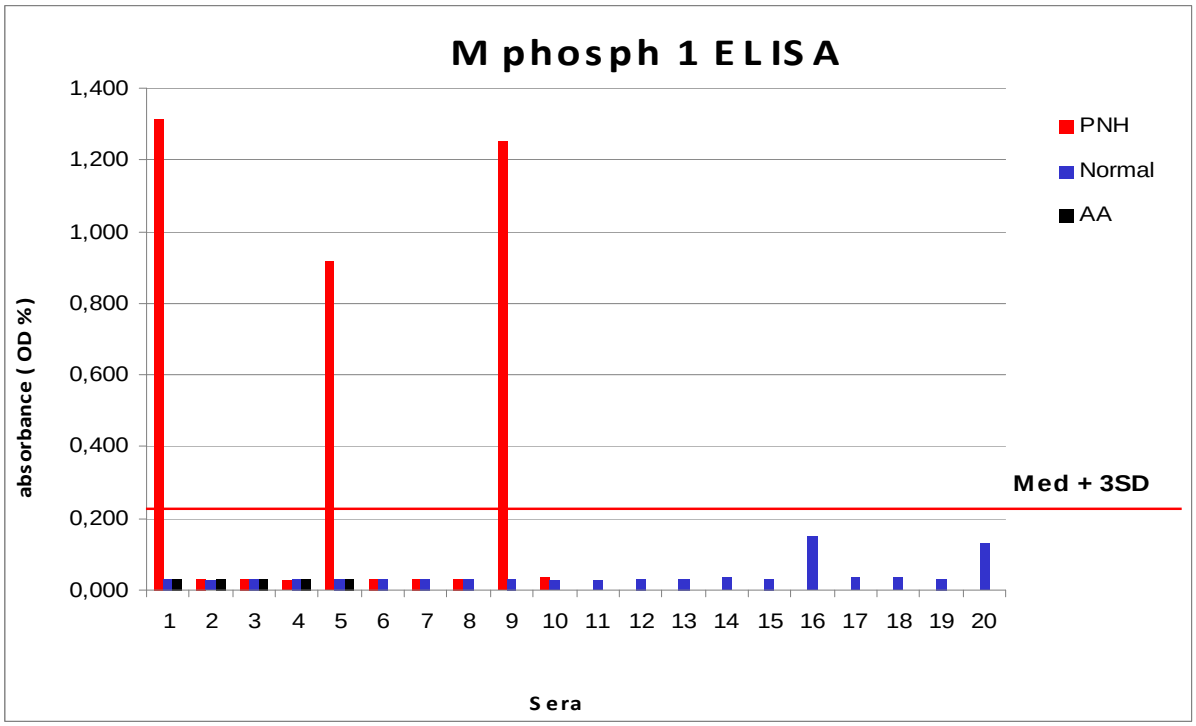


Figure 26: ELISA results of all sera against MPHOSPH1. With each of PNH sera number 1, 5 and 9 the optic density was higher than the medium levels of healthy donor + three times the standard deviation. With all sera from aplastic anemia patients and healthy donors, the density was lower than Med+3SD.

Desmoplakin reactivity was detected in four of the 10 PNH sera, but in none of the aplastic anemia sera. A positive reaction against desmoplakin was detected in one normal serum, and the OD_{600} of all positive PNH Sera (OD = 1.22 ± 0.07) was significantly higher than that of the normal serum (OD = 0.186 ± 0.09). Figure 27 presents the ELISA results of 10 PNH sera, 20 healthy donors and 5 aplastic anemia sera against Desmoplakin.

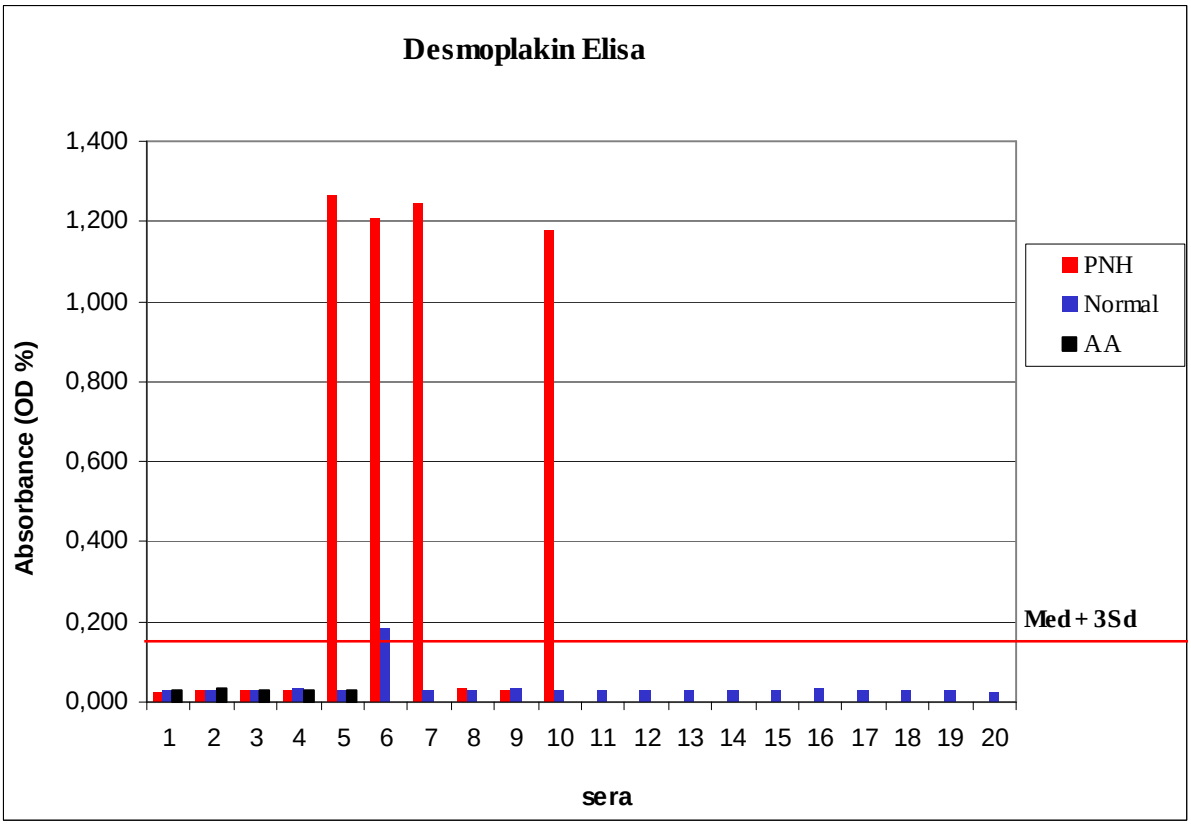


Figure 27: ELISA results of all sera against desmoplakin. With each of PNH sera number 5, 6, 7 and 10, the optic density was higher than the medium levels of healthy donor + three times the standard deviation. With all sera from aplastic anemia patients and healthy donors, the density was lower than Med+3SD.

5. Expression of desmoplakin and MPHOSPH1 can be identified by $CD34^+$ cells

The expression of desmoplakin and MPHOSPH1 genes by PCR was analyzed using cDNAs derived from testis and $CD34^+$ cells from one PNH patient and one healthy control. GAPDH was used as housekeeping gene. The gel electrophoresis of the PCR products is shown in (Figure 28). Both desmoplakin and MPHOSPH1 were expressed in $CD34^+$ cells of the PNH patient and normal individuals.

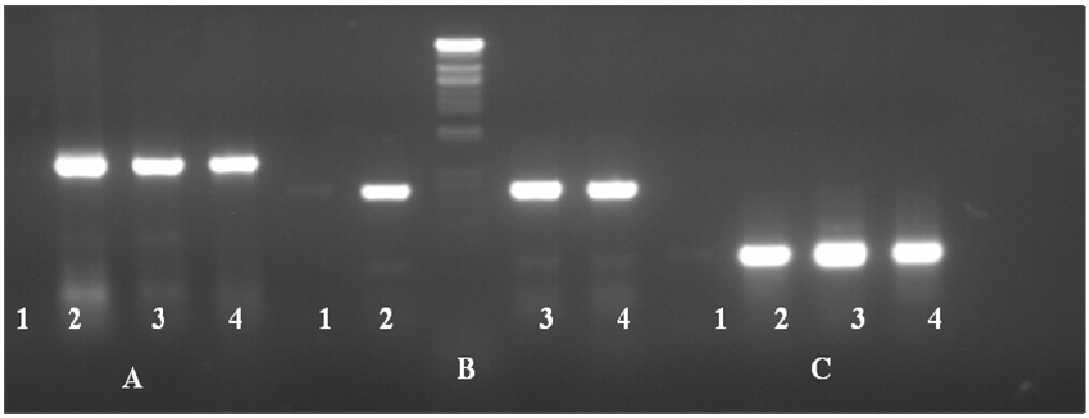


Figure 28: Gel electrophoresis analysis for cDNA from CD34$^+$ cells. A. Desmoplakin, B. MPHOSPH1, C. GAPDH. 1: Negative control, 2: Testis cDNA, 3: Normal CD34$^+$, 4: PNH CD34$^+$. The expression of Desmoplakin by normal (A3) and PNH patient (A4) could be identified. The expression of MPHOSPH1 also by normal (B3) and PNH (B4) patient is clear.

6. Epitope mapping

Using ELISA technique, the reactions of PNH sera against the DNA fragments of desmoplakin and MPHOSPH1 were tested. The reaction was considered positive if the absorbance value exceeded the mean absorbance value of that of healthy sera by three standard deviations. Positive reactivity against the epitopes number three and four (desmo-3 and desmo-4) for desmoplakin were documented in all PNH sera (5, 6, 7 and 10), that means that the epitope is the overlapping region, corresponding to aa's 1377 to 1387. The results of ELISA test are depicted in (Figure 29).

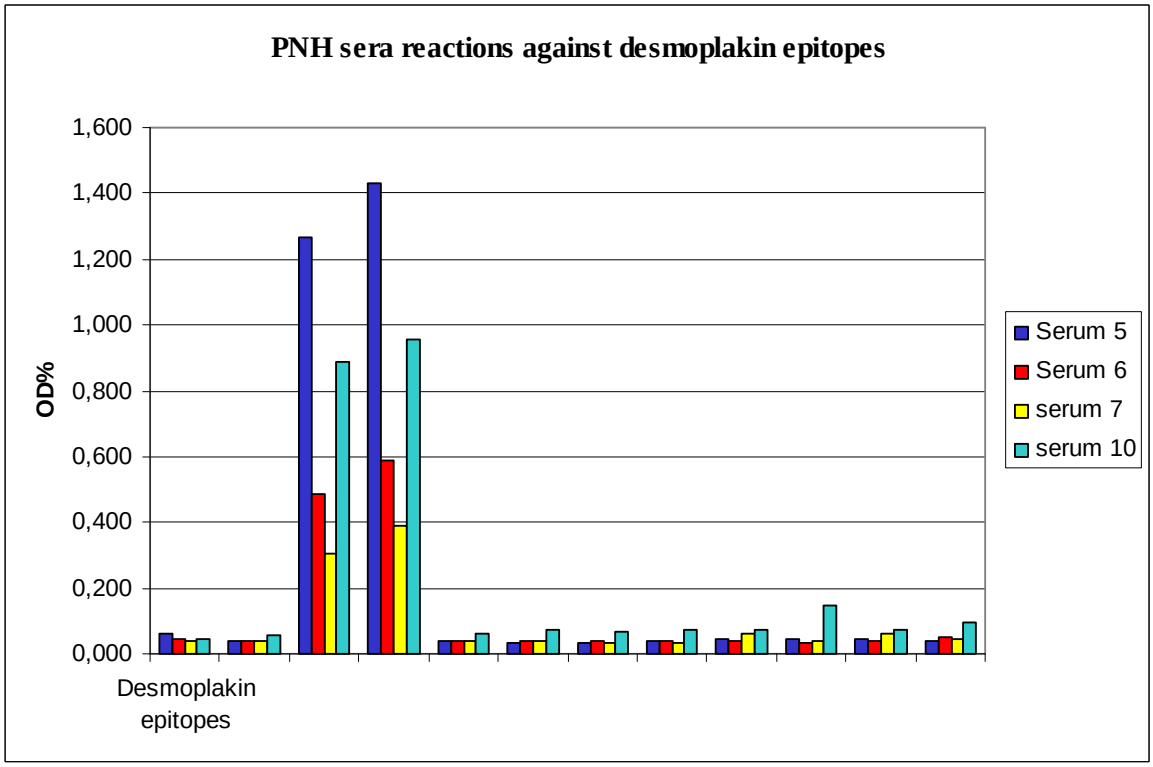


Figure 29: Elisa results of PNH sera against desmoplakin epitopes. PNH sera number 5, 6, 7 and 10 show reactivity against desmo-3 and desmo-4

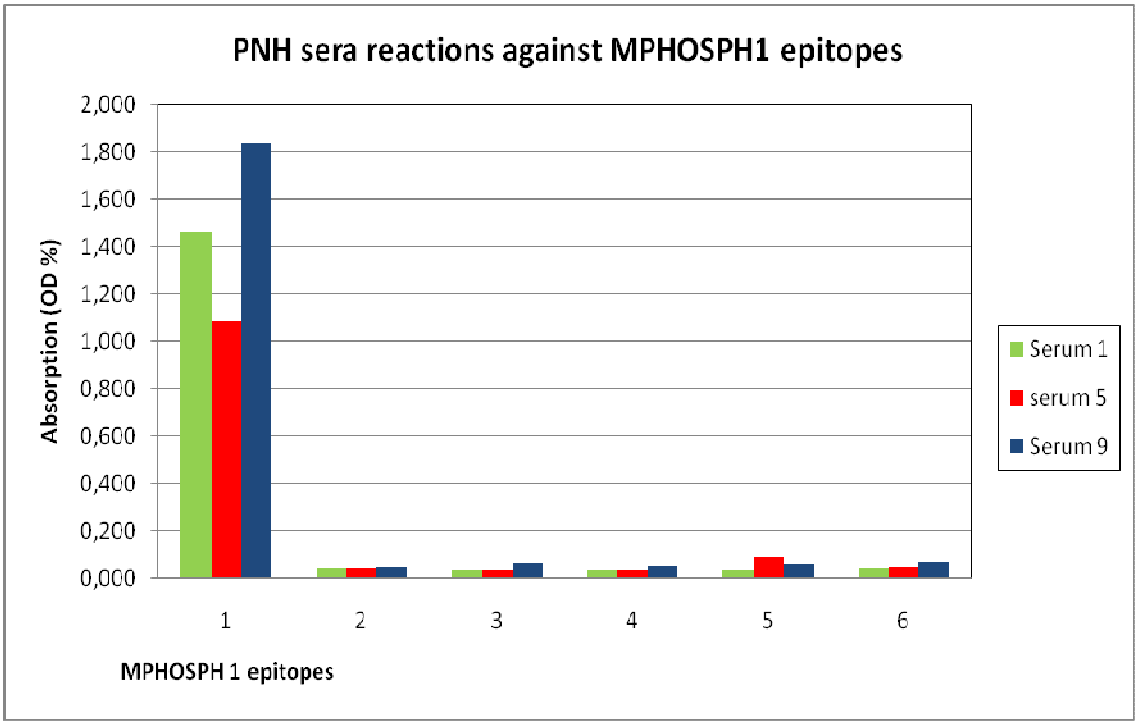


Figure 30: Elisa results of PNH sera against MPHOSPH1 epitopes. PNH sera number 1, 5 and 9 show reactivity against MPHOSPH1epitope number 1.

For MPHOSPH1, positive reactivity in PNH sera numbers 1, 5 and 9 were demonstrated against epitopes number 1 corresponding to aa's 204 to 321. The results are depicted in (Figure 30). The sequences of desmoplakin and MPHOSPH1 epitopes are shown in (Figure 31).

A. HQLTMQKEED
B. SHSIFTVKILQIEDSEMSRVIRVSELSLCDLAGSERTMKTQNEGERLRETGNINTSLL TLGKCINVLKNSEKSKFQQHVPFRESKLTHYFQ

Figure 31: Sequence of the epitopes. (A) Desmoplakin epitope. (B)) MPHOSPH1 Epitope.

VII. Discussion

Clonal expansion in PNH

PNH is a unique disorder characterized by the expansion of an abnormal clone or small number of clones, which replaces almost the entire hematopoietic stem cell pool. The pathophysiology leading to the expansion of GPI-deficient clones within the bone marrow of PNH or PNH/aplasia patients is still a matter of controversial discussions (Dunn *et al*, 1999). PNH clones do not exhibit a "malignant" characteristic, because PIG-A mutant clones occur at low levels in normal individuals without expanding or competing with normal hematopoietic cells (Araten *et al*, 2001). However, in PNH patients, clonal expansion can lead to a complete replacement of almost the entire hematopoietic stem cell pool in PNH patients. It was proposed that besides PIG-A mutations a second event enables the expansion of PNH clone over the residual normal hematopoietic cells (Inoue *et al*, 2006b). Genetic instability was identified in GPI-deficient cells present in PNH, which is also observed in related diseases with defined genetic aberrations such as MDS (Purow *et al*, 1999;Hattori *et al*, 1997). Such additional genetic alterations might affect the cellular activation, proliferation and differentiation program, which might be responsible for an altered growth behavior of GPI-deficient cells leading to clonal expansion in PNH. The most striking support for the hypothesis on additional genetic alterations within the GPI-deficient clone has been published In 1996. A chromosomal alteration in PNH clone has been documented in a Japanese patient with aplastic anemia who developed clinical evidence of PNH approximately 10 months after treatment (Nishimura *et al*, 1996). Only the PIG-A mutant cells (but not the GPI normal cells) were found to exhibit the chromosomal abnormality consisting of an insertion of a fragment derived from the long arm of one chromosome 12 into the long arm of the other chromosome 12; 46,XX, t(12;12)(q13;q15). Rearrangement of chromosome 12 was also documented in 2 patients with PNH (Inoue *et al*, 2006b). In both cases, der(12) had a break within the 3′ untranslated region of *HMGA2*, the architectural transcription factor gene deregulated in many benign mesenchymal tumors, that caused ectopic expression of *HMGA2* in the bone marrow (Grosschedl *et al*, 1994;Reeves, 2001). The ectopic expression in both patients appeared to be a consequence of gain-of-function mutational events caused by disruption of the 3′ UTR shown to contain elements that negatively regulate *HMGA2* transcription (Borrmann *et al*, 2001). However, the study neither established the sequence of events, which causes the clonal outgrowth of the double mutant cells nor presented an explanation how the

aberrant expression of *HMGA2* works with mutant *PIGA* to generate the proliferative phenotype.

Clonal immuno-selection in PNH

The frequent appearance of expanded GPI-deficient bone marrow cells in aplastic anemia patients has led to the hypothesis that GPI-deficient cells would escape the suppressive action of the immune system. This theory is further supported by the observation that in some PNH patients more than one GPI-deficient clone is present (Bessler *et al*, 1994d;Nafa *et al*, 1995). Whether a single PNH clone remains dominant or minor clones become dominant is still unclear. Furthermore, it is unknown how many hematopoietic stem cells (HSCs) sustain hematopoiesis and how long a single HSC can support hematopoiesis (Dunn *et al*, 1996). The relapse of PNH found in some patients after bone marrow transplantation (BMT) is associated with the appearance of new clones and not only the persistence of the original clones, which supports the concept that BM environment may create selective conditions favoring the expansion of PNH clones (Nafa *et al*, 1998).

In addition, the pathogenetic link between AA and PNH is intricate, but too apparent to be accidental. Previous studies indicated that circulating blood cells with a deficiency of GPI-anchored proteins could be detected in about 30–50% of the peripheral blood and bone marrow cells of some aplastic anemia patients (Kawaguchi *et al*, 1999;Schubert *et al*, 1994). This observation has led to the suggestion that normal hematopoietic stem cells might be damaged by the immunological mechanism. Whereas, the GPI-deficient cells are not attacked and therefore will grow out the damaged bone marrow environment. An immune selection/immune escape theory was previously proposed by Luzzatto's group (Bessler *et al*, 1994a). According to this theory, autoimmunity in AA is directed against either the GPI-anchor or a GPI-linked protein, PNH cells being spared, which expand and dominate the entire bone marrow. Luzzatto's idea implies the outgrowth of a dysregulated clone of immunocytes against GPI-linked proteins or their surface anchor. PNH clone(s) would thereby assume a growth advantage leading to their expansion in the aplastic marrow. The skewed T-cell repertoire in PNH patients is interpreted as the presence of specific T cell clones that recognize the GPI-anchor itself (Karadimitris *et al*, 2000;Karadimitris & Luzzatto, 2001b). T cells require additional signals besides that generated by the T cell receptor (TCR) complex to respond to antigens. Moreover, the interactions between co-stimulatory ligands and their receptors are critical for activation of T lymphocytes, prevention of tolerance and

development of T cell-dependent immunity (Kroczek *et al*, 2004). Therefore, by searching for the target antigen(s) of T cells in PNH, the concept that this target antigen(s) could be other than GPI-anchor antigen, and a GPI-anchor protein(s) just works as co-stimulatory factor for the activated T cells, should be considered (Murakami *et al*, 2002d).

Because the expression of GPI anchor is not restricted to HSC, an autoimmune attack against the GPI anchor would probably cause fatal ubiquitous tissue damage. Moreover, the concept that the GPI anchor serves as the target of immune system, means that PNH cells lacking the GPI-anchor are more resistant to T cell attack than normal (GPI^{+}) cells. However GPI-deficient cells exhibit the same susceptibility to the cytotoxic action of allogeneic T-cells as normal cells (Karadimitris & Luzzatto, 2001a). Alternatively, it has been proposed that the autoimmune attack restricted to HSC could be associated with an expansion of cytotoxic T/NK cells recognizing GPI-anchor structures in the context of CD1d molecules. Such clones were found in other autoimmune diseases, but not in PNH or AA patients so far. An alternative explanation could be that GPI-linked surface proteins such as ULBP's (cytomegalovirus glycoprotein UL16 binding proteins) might directly interfere with activating receptors (NKG2D) on NK cells (Pende *et al*, 2001). However, the expression pattern of these molecules is unknown to date.

It has been also suggested that GPI-deficiency itself leads to an increased resistance to apoptosis (Brodsky *et al*, 1997). Moreover, it was documented that granulocytes not only from PNH, but also from AA and MDS patients are relatively resistant to apoptosis (Nagakura *et al*, 2002b). If GPI-deficient cells would exhibit a resistance to apoptosis only because of the absence of GPI-linked surface molecules, clonal dominance of the GPI-deficient clone would be found also in AA and MDS. However, such a resistance strictly due to GPI-deficiency could not be reproduced by other groups (Ware *et al*, 1998;Bastisch *et al*, 2000a). Therefore, it is suspected that resistance to apoptosis observed in GPI-deficient cells of PNH patients would be a result of changes distinct from the deficient surface expression of GPI-linked proteins.

Based on all observations mentioned above, the immune selection/immune escape theory can give a better explanation of both the GPI deficient cells documented in aplastic anemia and the clonal dominance in PNH. However, the target antigens of such immune attack have to be identified in order to understand the mechanism of this selection. The alternative hypothesis, as mentioned above, includes internal alterations within clonal cells such as mutations in

genes regulating cell proliferation and apoptosis. A combination between both theories might provide a basis for more understanding of the pathophysiology of PNH, so that; the development of PNH will need three steps, elucidated in (Figure 32):

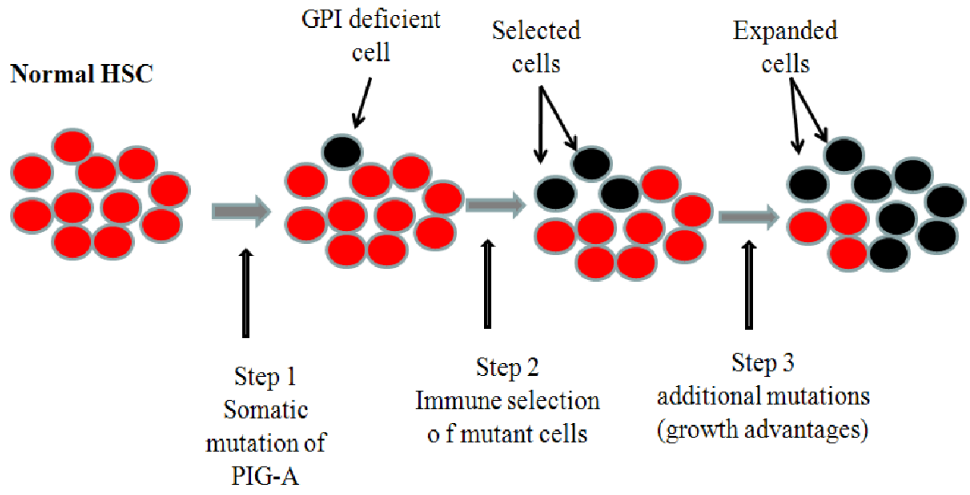


Figure 32: Sequence of events necessary for development of PNH. Modified according to (Inoue *et al*, 2006b)

In the first step, a somatic mutation of PIG-A gene on the X chromosome occurs. The second step will be the immune attack directed against normal HSC, which spares the GPI $^{-}$ cells because either these cells are not expressing the target antigens of the immune system or because the deficiency of some GPI-anchor proteins makes these cells less susceptible to be killed by cytotoxic T cells. This hypothesis would be supported by the observation that in the context of aplastic anemia, the proportion of GPI-deficient cells expands to a certain extent, but in most cases does not give rise to a predominant clone within the bone marrow. In the third step, an additional mutation provides the internal growth advantage to the selected GPI $^{-}$ cells in order to expand and fill the vacuum caused by the immune attack of normal cells. The sequence of events in clonal expansion could be explained more adequately by a combination of both opposed hypotheses.

This hypothesis implies the possibility of autoantibody production by PNH patients. A defect in the immune system in the second step leads to the production of antibodies and consequently an immune attack directed against special antigens. The identification of these antigens could help to understand the exact mechanism of the immune selection in PNH. Moreover, the identification of a humoral immune response to the target antigens may imply a pathologic T-cell immune response to the same antigens. According to the clonal expansion in the third step, the immune system recognizes the genetic alterations in the target cells and

produces antibodies against the modified antigens. Although these antibodies might play no role in the pathophysiology of the disease, the identification of these antibodies will prove the occurrence of such alterations in the target cells.

In order to identify the target antigen, the SEREX approach was applied to screen a human fetal liver cDNA library, because fetal liver is known to be highly enriched for $CD34^+$ cells. Using sera from 10 PNH patients, two auto-immunogenic structures were found: MPHOSPH1 (known also as kinesin family member 20B or KIF20B) and desmoplakin (DSP).

M- Phase phosphoprotein 1 (MPHOSPH1)

The human M-phase phosphoprotein 1 (MPHOSPH1) was originally identified through a screening of a subset of proteins specifically phosphorylated at the G2/M transition (MatsumotoTaniura *et al*, 1996a). MPHOSPH1 is a 225 KD protein, characterized as a plus-end-directed kinesin-related protein. Recombinant MPHOSPH1 exhibits in vitro microtubule-binding and microtubule-bundling properties as well as microtubule-stimulated ATPase activity. Using polarity-marked microtubules it was found that MPHOSPH1 is a slow molecular motor that moves toward the microtubule plus-end at a 0.07 μm/s speed. In cycling cells, MPHOSPH1 localizes mainly at the nuclei in interphase. During mitosis, MPHOSPH1 is diffused throughout the cytoplasm in metaphase and subsequently localizes to the midzone to further concentrate on the midbody. MPHOSPH1 suppression by RNA interference induces failure of cell division late in cytokinesis. Autoantibodies against MPHOSPH1 were found in 40% of patients of idiopathic ataxia, which is known to be an autoimmune disease (Fritzler *et al*, 2000). In this study, IgG antibodies against MPHOSPH1 were found in 3 out of 10 PNH patients (30%). No antibody response against MPHOSPH1 could be detected in 10 MDS patients, 5 aplastic anemia patients, or 20 normal individuals. Occurrence of anti-MPHOSPH1 Abs in PNH patients and none of MDS or aplastic anemia patients supports a specific association of these antibodies with PNH. However, it should be mentioned that these antibodies were documented in a part and not all PNH patients included in the study.

Moreover, the expression of MPHOSPH1 in $CD34^+$ cells (from PNH and normal individual) was demonstrated in the study, which gives more support to the theory that MPHOSPH1 could be a target antigen of the immune attack in PNH. In addition to their expression in human hematopoietic cells, MPHOSPH1 transcripts were previously found in human Kidney, testis, and brain (Abaza *et al*, 2003a).

The sequencing of MPHOSPH1 DNA from PNH $CD34^+$ cells and normal $CD34^+$ cells revealed no differences in the sequences, however posttranslational modifications could not be ruled out. Although, the nuclear localization of MPHOSPH1 protein during the cell cycle may undermine the theory that anti-MPHOSPH1 antibodies affect the function and viability of hematopoietic cells in PNH patients. It was documented in other studies (Bastisch *et al*, 2000b) that anti-MPHOSPH1 antibodies occur in a high frequency with other autoimmune diseases (40 % of idiopathic ataxia patients) and play a role in the pathophysiology of this autoimmune disease. Furthermore, it was documented that the immune reaction, for example in a solid tumor, can be directed against intracellular antigens (Yoon & Jun, 1999b). Thus, MPHOSPH1 could be a candidate antigen, which evokes the reaction of immune system against hematopoietic stem cells, especially because the expression of the protein in $CD34^+$ cells was documented in this study.

Desmoplakin

Desmoplakin is a (240 KD) protein associated with desmosomes. Desmosomes are intercellular junctions that tightly link adjacent cells and desmoplakin is an obligate component of functional desmosomes that anchor intermediate filaments to desmosomal plaques. The N-terminus of desmoplakin is required for localization to the desmosome and interacts with the N-terminal region of plakophilin 1 and plakoglobin. The C-terminus of desmoplakin binds to intermediate filaments. In the mid-region of desmoplakin, a coiled-coiled rod domain is responsible for homodimerization (Arnemann *et al*, 1991). Autoantibodies against desmoplakin were found not only in patients with paraneoplastic pemphigus (Anhalt *et al*, 1990), but also in patients with non-neoplastic pemphigus (Jiao & Bystryn, 1998a;MatsumotoTaniura *et al*, 1996a) which means that, the production of these antibodies is not necessarily tumor-related. Although the roles of anti-desmoplakin antibodies in the pathophysiology of paraneoplastic pemphigus are not known, their existence is one of the diagnostic criteria for the disease (Oursler *et al*, 1992). Serological activity against desmoplakin was demonstrated in 4 out of 10 classic PNH patients (40%), but none of the 10 MDS patients or 5 aplastic anemia patients exhibited reactivity against desmoplakin. In a 1/100 diluted normal serum, a weak reactivity against desmoplakin could be identified; however the reactivity disappeared after dilution of the serum up to 1/ 1000. Moreover, determination of anti-desmoplakin antibodies with ELISA indicated that the OD of all positive PNH Sera (OD = 1.22 ± 0.07) was significantly higher than that of the normal serum (OD = 0.186 ± 0.09). The expression of desmoplakin was demonstrated in $CD34^+$ cells from

both normal and PNH bone marrow. The sequences of desmoplakin cDNA from the patient and the control were identical. Although the localization of desmoplakin is intracellular and the expression of the protein is ubiquitous in the body tissues, the role of anti-desmoplakin antibodies in the pathophysiology of auotimmune disease (e.g. pemphigus) could not be determined. The serological reactivities against MPHOSPH1 and desmoplakin were documented in patients who did not have a history of transfusion (patients number 1 and 7 exhibited reactivity against MPHOSPH1 and desmoplakin respectively) and other patients who had received transfusion (patient number 5), therefore it is unlikely that the patients developed antibodies because of the blood transfusion.

The results of the study support the hypothesis that humoral immune responses to antigens on hematopoietic cells are involved in the pathophysiology of PNH. The finding of high-titered IgG responses found in PNH patients implies cognate T-cells help. In an approach of "reverse T-cell immunology", it is now possible to determine the epitopes of MPHOSPH1 and desmoplakin that are presented to $CD4^+$ and $CD8^+$ cells in the context of MHC-II and MHC-I epitopes respectively. The elucidation of these T-cell responses will give us more insight into the role of anti-desmoplakin and anti-MPHOSPH1 in the pathophysiology of PNH. Analysis of larger numbers of patients with PNH will show whether anti-MPHOSPH1 and anti-desmoplakin antibodies can be used as diagnostic and/or prognostic markers of PNH. While MPHOSPH1 and desmoplakin are expressed by $CD34^+$ cells in both PNH patients and healthy individuals, the conditions that render the two structures immunogenic in patients with PNH, but not in healthy controls, must still be determined. According to one model, the presentation of non-altered molecules in the context of "danger" breaks down auto-tolerance (Matzinger, 2002). Alternatively, mutations of the two antigens in the $CD34^+$ cells of PNH patients, or posttranslational modifications like differential glycosylations or phosphorylations could render desmoplakin and MPHOSPH1 auto-immunogenic in PNH patients. The exact role of the isolated antigens in the pathophysiology of PNH has not yet been definitely demonstrated. So far, it has not yet been completely excluded that these antibodies may arise by chance. However, the titers obtained from the patients compared to the controls are quite striking. In addition, it might be argued that the antibodies are not consistently present in all PNH patients. So far, it is not yet clear what the immunological mechanism of clonal expansion consists of. It may be that the immunological basis of such an immunological recognition is quite heterogeneous and consists of multiple antigen recognition mechanisms. It is of course very challenging to assume a pathophysiological role for the detected antigens. On the other hand, in the case of autoimmunity, target structures would be of interest as was

shown for patients with aplastic anemia. In autoimmune disorders involving solid tissues, proteins likely to elicit autoimmune reactions are `cryptic', i.e. intracellular, rather than expressed at the surface, and presented to the immune system upon disintegration of the cell (Atkins *et al*, 2000;Yoon & Jun, 1999a).

Finally, such antigens detected by autoantibodies could serve as a guide for deregulated genes in affected cells. The specificity of desmoplakin and MPHOSPH1 as auto antigens for PNH must be further investigated by studying another group of autoimmune diseases. Until then, desmoplakin and MPHOSPH1 remain the most promising proteins in the immuno-pathophysiology of PNH.

Immune response in MDS patients

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired neoplastic disorders of multipotent hematopoietic stem cells characterized by increasing bone marrow failure with quantitative and qualitative abnormalities of all three myeloid cell lines. A hallmark of the disease is an active but ineffective hematopoiesis leading to peripheral pancytopenia. The pathophysiology of bone marrow failure by MDS patients, which causes blood transfusion dependence and neutropenic infection, is still unknown. MDS share some of the features of aplastic anemia, a disease with an established autoimmune pathogenesis, which suggests a role of the immune system in the pathophysiology of bone marrow failure in MDS similar to that in aplastic anemia. Many investigations focused on the immune-mediated bone marrow failure especially in the early stages of MDS as in refractory anemia RA and refractory anemia with ringed sideroblasts RARS, (Barrett *et al*, 2000). Cases of MDS showed also response to immune suppressive therapy (IST) with anti-thymocyte globulin, which is known to be effective in aplastic anemia. This therapy is associated with loss of lymphocyte-mediated inhibition of granulocyte-macrophage colony-forming units and alterations in T-cell receptor Vβ profiles (Molldrem *et al*, 1998). Different antibodies were found in bone marrow culture of many MDS patients, especially in RA patients, but neither the role of these antibodies in cytopenia nor the target antigens of these antibodies could be identified. Many clinical observations and laboratory studies suggest that the immune system might mediate bone marrow failure in some MDS patients. In one respect, the suggestion depended on the accumulating evidence of immunologic abnormalities in MDS, including, high levels of tumor necrosis factor (TNF, Gersuk *et al*, 1998), differentially regulated FLIP (FAS-associated death domain-like interleukin [IL]-1 converting enzyme, Kochenderfer *et al*,

2002a), presence of cytotoxic T cells and clonality of T-cell clones (Maciejewski *et al*, 2002). On the other hand, the conclusion that the immune system may mediate bone marrow failure in some MDS patients is underscored by the association between MDS and many autoimmune diseases (Hamblin, 1987): MDS is sometimes seen in conjunction with Reynaud's syndrome, rheumatoid arthritis, and polymyalgia rheumatica. Furthermore, MDS is associated with aplastic anemia: patients with AA can develop MDS and have overlapping syndromes of severe aplastic anemia with features of MDS, which frequently give rise to diagnostic confusion. Tichelli et al, were the first to describe inhibition of erythroid colony growth by autologous T cells in some patients with MDS (Tichelli *et al*, 1988). After that, many studies demonstrated that treatment with anti-lymphocyte globulin (ATG) abrogates the T-cell mediated suppression of granulocyte colony growth in MDS patients, which improves marrow function and lessens transfusion dependence (Barrett & Sloand, 2009). These findings strongly support T-cell mediated marrow suppression as the cause of cytopenia in about 20–30% of MDS patients.

Despite all immunological findings in MDS patients (which may indicate an alteration in regulatory mechanisms of B lymphocytes) (Okada *et al*, 1999), the exact role of these abnormal immune reactions in the etiopathogenesis of MDS is not known. It was suggested that an increase in the number of monocytes, which is frequent in all types of MDS, explains the alteration, for example, monocytes secrete IL-1 that regulates the growth and differentiation of B lymphocytes (Lipsky *et al*, 1983). On the other hand, many patients with MDS receive multiple transfusions and a significant percentage of them become alloimmunized against RBC antigens (Sokol *et al*, 1989). Moreover, in some MDS patients, there is a high incidence of red cell antibodies without clinical or laboratory alteration in hemolysis (Novaretti *et al*, 2001). In this study, the serological response against hematopoietic stem cells in a cohort of MDS patients was tested in the same way as the PNH patients included in the study. Ten patients with MDS were screened using the SEREX approach with a human fetal liver cDNA library and a cDNA library from $CD34^+$ cells of a normal individual. No reaction, neither by MDS pool nor by each individual serum could be identified using both the human fetal liver cDNA library and the normal $CD34^+$ cells cDNA library. My findings support the suggestion that PNH and MDS are separate diseases and not one syndrome. While no serological reactions could be identified in MDS sera against hematopoietic cells, such reactions were documented by the screening of PNH sera, which postulates an immune reactivity in the pathophysiology of some PNH patients in form of antibody response against MPHOSPH1 and/or desmoplakin. Further studies are required in

order to determine the mechanism by which desmoplakin and MPHOSPH1 evoke the immune system in a larger number of PNH patients. Analysis of the structure of both proteins in $CD34^+$ cells from PNH patients, the type of HLA on HSPCs that presents the antigens to the T cells, and the ability of the peptides derived from both antigens to activate cytotoxic T cells should explain the role of these antigens in the pathophysiology of PNH. Moreover, determination of changes in the level of antibodies against both antigens in sera of PNH patients, during the treatment and after bone marrow transplantation may give more information about the usefulness of using these antibodies as diagnostic and/or prognostic markers in PNH.

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