Effect Of Complement Regulatory Proteins In RBC Lysis

A Dissertation Thesis Submitted To

Institute of Science, Nirma University,

In Partial Fulfilment

for the Degree of

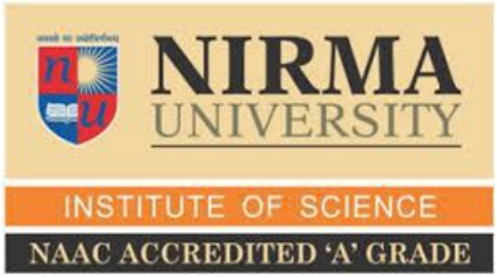
MASTER OF SCIENCE

Submitted by,

Ankita Oza (18MBT001)

Guided by,

Dr. Nasreen Munshi and Mr. Pankaj Kalita



Institute of Science
Nirma University
Sarkhej Gandhinagar Highway, Ahmedabad – 382481

April 2020

DECLARATION

I hereby kindly declare that the work titled **"Effect of Complement Regulatory proteins in RBC Lysis**" is my original work. I have not copied from any other student's work or from any other sources except where due references or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

Date:

Ankita Oza

ACKNOWLEDGEMENT

"Basic research is like shooting an arrow in the air, where it lands, painting a target"

-Homer Burton Adkins

It is said that success comes to those who believe in it and have that passion and determination to achieve it. I am extremely thankful to Prof. Sarat Dalai, Director, Institute of Science, Nirma University for providing the necessary resources to accomplish the research work and giving me this opportunity.

This project required a lot of guidance and I am grateful towards my guide Dr. Nasreen Munshi and Mr. Pankaj Kalita from Zydus Research Centre for providing their support and encouragement throughout the path of research work.

I would like to pay peculiar thanks to Mrs. Rutu Upadhyay, Ms. Sneha Patel and Ms. Narayani Vyas for always being there as a considerate hand. I would also like to thank the Ph.D. scholars, Ms. Priya Patel and Mrs. Anwesha Mukherjee for their valuable support.

Also a decisive thanks to all faculty members namely Dr. Shalini Rajkumar, Dr. Vijay Kothari, Dr. Amee Nair, Dr. Dablulal Gupta, Dr. Kuldeep Verma, Dr. Sonal Bakshi, Dr. Sriram Sheshadri and Dr. Pranati Sar for their critical reviews.

Valuable thanks to staff support at both Nirma University and Zydus Research Centre including, Mr. Sachin Prajapati, Mrs. Sweta Patel, Mr. Karshanbhai Solanki and Mr. Chandubhai Jadav for lending all the lab requirements. Also, Mr. Rajendra Patel, Mrs. Svetal Shukla and Mr. Hasit Trivedi are acknowledged for timely support.

_____

Ankita Oza

INDEX

Sr. no.	Title	Page no.
1.	Introduction	08
1.1	Classical Pathway	11
1.2	Lectin Pathway	14
1.3	Alternative Pathway	16
1.4	Diverse function of complement	19
1.5	Regulation of the complement system	21
1.6	Dysregulation of the complement system	26
2.	Rationale of Study	28
3.	Objective	30
4.	Materials and Methods	32
4.1	Materials	33
4.2	Simulation of the PNH condition in the blood cells	36
4.3	Methods	37
4.3.1	Standardization Assay	37
4.3.2	C3b Deposition Assay	37
4.3.3	Lysis Assay	38
5.	Results and Discussion	39
6.	Conclusion	59
7.	References	61

ABBREVIATIONS

RBC: Red Blood Cells

WBC: White Blood Cells

MAC: Membrane Attack Complex

MBL: Mannose Binding Lectin

MASP: MBL- Associated Serine Proteases

DAF: Decay Accelerating Factor

MCP: Membrane Cofactor of Proteolysis

CD: Cluster of Differentiation

PNH: Paroxysmal Nocturnal Hemoglobinuria

GPI: Glycosyl Phosphotidyl Inositol

GVB: Gelatin Veronal Buffer

EGTA: Ethylene glycol-bis (2-aminoethyl ether) tetra acetic acid

PBS: Phosphate Buffered Saline

FACS: Fluorescence Activated Cell Sorting

LIST OF TABLES

Table no.	Table Title	Page no.
1	Composition of Alsever Buffer	33
2	Composition of Veronal Buffer	33
3	Antibodies Used	34
4	Composition of 1X PBS	35
5	Controls used in the Standardization Assay	40
6	Samples used in the C3b deposition Assay	49
7	Composition of wells in the lysis plate	58

LIST OF FIGURES

Figure no.	Figure Name	Page no.
1	The classical pathway	11
2	Lectin pathway	14
3	Alternative pathway	16
4	Dysregulation of the Complement System	26
5-12	FACS results of Standardization assay	41-48
13-18	FACS results of C3b deposition assay	50-55
19	Lysis Assay and depiction of button formation at the base of the well is observed due to accumulation of the whole RBCs.	57

Introduction

1. INTRODUCTION

Blood is the body fluid in humans and many animals. It delivers necessary substances such as nutrients and oxygen to the cells and transports metabolic waste products away from the same cell. There are antibodies also present in the blood which protect us from infections. Blood also has an important role in maintaining homeostasis. Blood is composed of plasma, Red Blood Cells (RBC), White Blood Cells (WBC), and platelets. Red Blood Cells (RBC) are the major highlight here. RBCs generally have a lifespan of about 120 days and after that they are lysed or get degraded. New RBCs are formed by the bone marrow in our body at particular time intervals. In the process of lysis of the old RBCs comes the role of the complement system or complement proteins. The word **complement** refers to a set of serum proteins that cooperate with both the innate and adaptive immune systems to eliminate the blood and tissue pathogens. The complement proteins generally work in the form of a catalytic cascade. Research work on the complement started in the 1890s by Jules Bordet at Institute Pasteur in Paris. His experiment showed that when sheep antiserum was added to the bacterium *vibrio cholera* it caused the lysis of the bacteria and heating the antiserum to destroy the bacteriolytic activity. But the ability to lyse the bacteria was restored to the heated serum by adding fresh serum that contained no Antibacterial Antibodies. This led him to a conclusion that bacteriolysis requires two different substances: the heat-stable specific antibodies that bound to the bacterial surface, and a second, heat-labile (sensitive) component responsible for the lytic activity. So to purify the heat-labile component Bordet developed antibodies specific for red blood cells and identified the antibodies that induce lysis of the red blood cells also known as hemolysis. Paul Ehrlich also carried out a similar experiment and coined the term **complement defining it as "the activity of blood serum that completes the action of Antibody".** Complement components are classified in seven categories:

1. Initiator complement components
2. Enzymatic mediators
3. Membrane-binding or opsonins
4. Inflammatory mediators
5. Membrane attack proteins

6. Complement receptor proteins
7. Regulatory complement components

The classical pathway comprises of 3 pathways which are:

a. Classical Pathway
b. Lectin Pathway
c. Alternative Pathway

1.1 CLASSICAL PATHWAY

Classical pathway of complement activation is considered to be the part of the adaptive immune response as it begins with the formation of the Antigen-Antibody complexes (Figure 1).

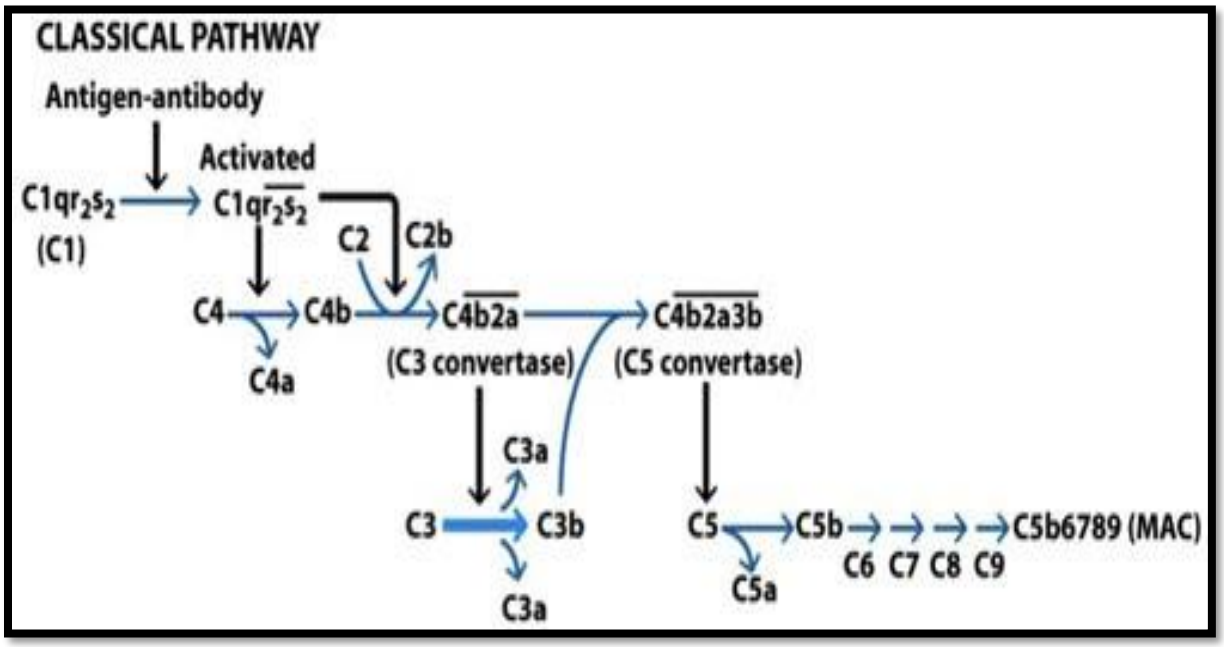


Figure 1: The classical pathway.

(http://csir-lifescience.blogspot.com/2011/09/complement-system.html**)**

Complexes formed by IgM or certain sub-classes of IgG Antibodies are capable of activating the classical complement pathway. Initial stage of activation involves the complements C1, C2, C3 and C4, which are present in plasma as zymogens. Antigen-antibody complex induces a conformational change in the non-antigen binding region (Fc). This conformational change exposes a binding site for the C1 component of complement.

INITIATION

C1 exists as a macromolecular complex **consisting of one molecule of C1q and 2 molecules each of the serine proteases, C1r and C1s.** All of them are held together in a Ca^{2+} stabilized complex ($C1qr_2s_2$).

C1q

C1q is composed of 18 polypeptide chains. It associates to form six collagen-like triple helical arms, at tip of which is CH2 domain of antibody molecule. Each C1 macromolecular complex must bind by its C1q globular heads to atleast two Fc sites for a stable C1-Antibody interaction. The circulating and non-antigen bound IgM adopts a planar configuration. In this C1q binding sites are not exposed. But when the IgM is bound it forms a staple structure. Stapled peptides consist of peptide chains that bring an external brace that force the peptide structure into an *a*-helical one (*Moiola et.al.,2019*). This staple structure has atleast three binding sites exposed for C1q. In case of IgG it contains only one C1q binding site per molecule. Now when the C1q binds to the CH2 domain of the Fc region it induces a conformational change in one of the C1r molecules that converts it to an active serine protease enzyme. The C1r molecule then cleaves and activates the other C1r molecule. The two C1r proteases then cleave and activate the other C1r molecule.

C1s

C1s has two substrates, C4 and C2. C1s hydrolyzes C4 component into C4a (it is an anaphylatoxin) and C4b (attaches covalently to the target membrane surface in the vicinity of C1, and then binds C2). C4b binding occurs when an unstable, internal thioester bond is exposed on C4b, reacts with hydroxyl or amino groups of proteins or carbohydrates on the cell membrane. This reaction happens quickly otherwise the C4b is further hydrolyzed and can't form covalent bonds with the cell surface (approximately 90% of c4b is hydrolyzed before it binds to the cell surface). C4b binds to C2 and exposing it to the action of C1s. C1s cleaves C2. On binding of C4b to C2, it becomes susceptible to cleave the neighbouring C1s enzyme. C2b diffuses away. An **enzymatically active complex C4b2a**

is formed. C2a is an enzymatically active fragment but it remains active only when bound to C4b.

AMPLIFICATION

C4b2a is also known as C3 convertase. The C3 convertase hydrolyzes C3 into C3a (a small anaphylatoxin) and C3b (tremendous amplification of this molecule occurs and it also binds to the microbial surface, providing a molecular "tag"). The C3b further generated is very important in many actions of complement. C3b possesses following properties:

- The presence of molecular tag allows the phagocytosis of cells and engulfs the tagged microbes, also known as **Opsonization.**
- They can attach to the Fc portion of antibodies participating in soluble antigen-antibody complexes.

  These C3b tagged immune complexes are bound by C3b receptors on phagocytes or RBC. They are either phagocytosed or conveyed to the liver where they are destroyed.
- It is also required for the formation of C5 convertase.

C4b2a3b forms the trimolecular membrane bound complex known as C5 convertase.

TERMINATION

C5 convertase then hydrolyzes the C5 component into C5a and C5b. The C5a diffuses away and C5b activates the C6, C7 and C8 components. They form a complex structure and further activate C9 component. The activation of all these components leads to the Membrane Attack Complex (MAC) formation. MAC leads to the formation of pores in the membrane and this causes osmotic imbalance and the cells lyse.

1.2 LECTIN PATHWAY

This pathway uses lectins. Lectins are proteins that recognize specific carbohydrate components primarily found on microbial surfaces as its specific receptor molecules. Mannose binding lectin (MBL) is used as an initiator in this pathway (Figure 2).

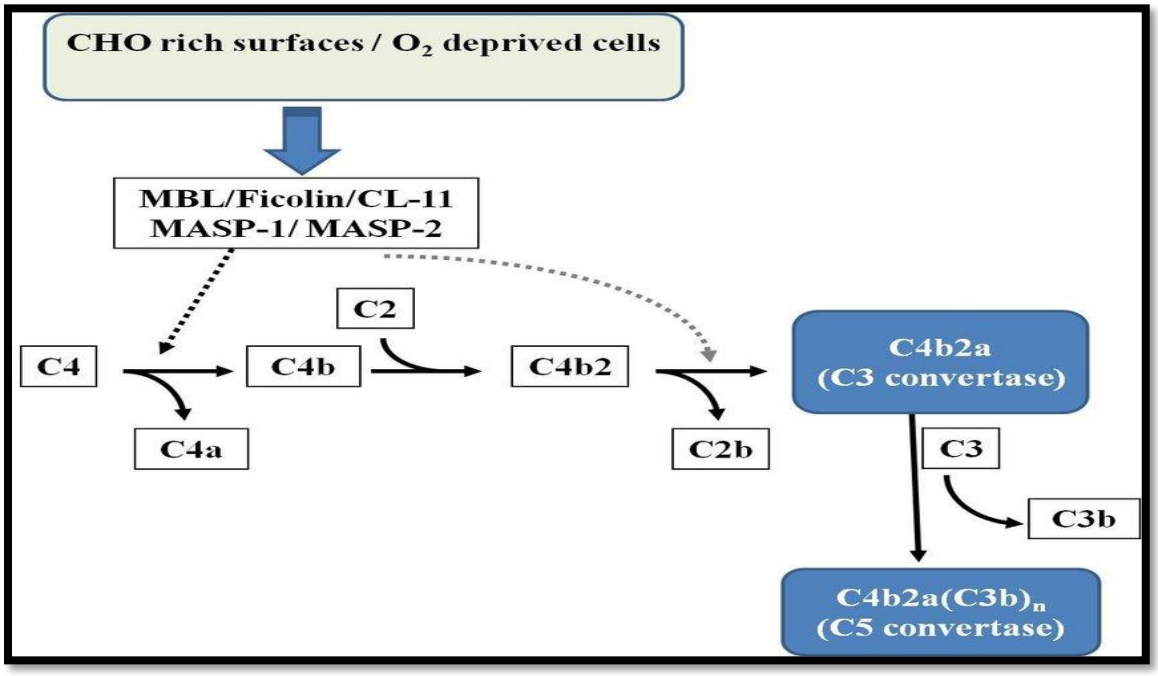


Figure 2: Lectin Pathway.

(https://www.researchgate.net/figure/Lectin-pathway-activation-on-Pathogen-Associated-Molecular-Patterns_fig4_314213249**)**

INITIATION

Mannose Binding Lectin (MBL) is the first lectin demonstrated to be capable of initiating complement activation. It binds to the close knit array of mannose residues found on microbial surfaces of bacterial strains (like: *Salmonella, Listeria* and *Neisseria),* strains of fungi (like *Cryptococcus neoformans* and *Candida albicans*) and some viruses (HIV-1 and respiratory syncytial virus). It also recognizes various structures in addition to mannose including N-acetyl glucosamine, D-Glucose and L-Fucose as all of them have associated

hydroxyl group. It is the classic pattern recognition receptor. It is observed that low levels of MBL causes serious bacterial infections. They are expressed in the liver and resemble C1q structurally. It belongs to the subclass of lectins known as collectins.

Recently, Ficolins another family of protein structure related to collectins, have been recognized as additional initiators. L-Ficolin, H-Ficolin and M-Ficolin each binds to specific types of carbohydrates on microbial surfaces. MBL is associated in the serum with MBL-Associated Serine Proteases or MASP proteins. Three MASP proteins have been identified: MASP1, MASP2 and MASP3. Out of these MASP2 is the most important one.

MASP2

MASP2 is structurally related to the serine proteases C1s, and can cleave both C2 and C4 forming the C3 convertase. It utilizes the same components as classical pathway with the single exception of the C1 complex. Lectin replaces the antibody as the antigen-recognizing component. MASP proteins take the place of C1r and C1s in cleaving and activating the C3 convertase.

AMPLIFICATION

After the formation of C3 convertase, it then hydrolyzes the C3 component into C3a and C3b. C3a diffuses away. C3b formed is very important for the further steps, as C3b further combines with the complex and forms C5 convertase.

TERMINATION

C5 convertase then hydrolyzes the C5 component into C5a and C5b. The C5a diffuses away and C5b activates the C6, C7 and C8 components. They form a complex structure and further activate C9 component. The activation of all these components leads to the Membrane Attack Complex (MAC) formation. MAC leads to the formation of pores in the membrane and this causes osmotic imbalance and the cells lyse.

1.3 ALTERNATIVE PATHWAY

Alternative Pathway is considered to be a part of the innate immune system (Figure 3).

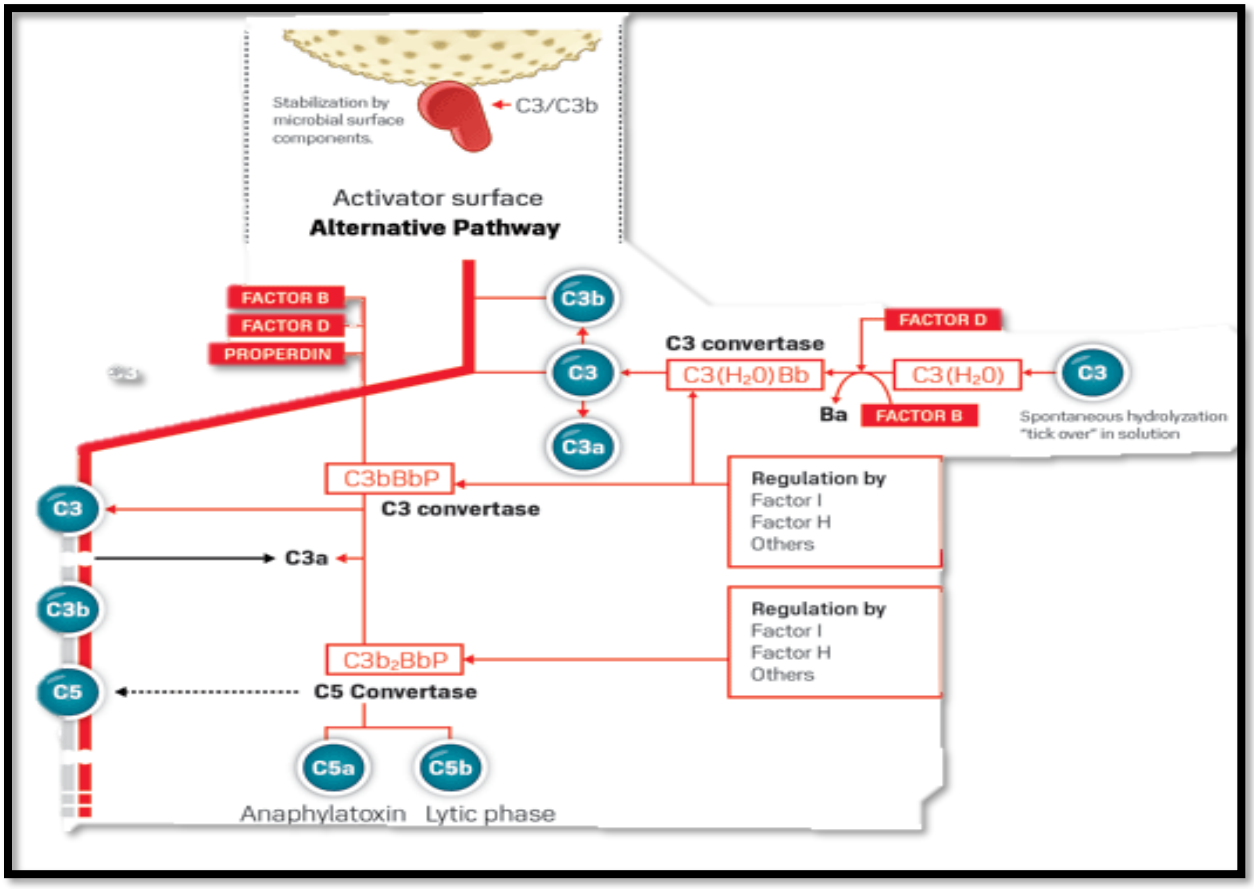


Figure 3: Alternative pathway.

(http://www.complementsystem.se/alternative-pathway**)**

It uses its own set of C3 and C5 convertases. In this pathway C3 convertase is made up of one molecule of C3b and one unique molecule Bb. One another C3b is added in order to form C5 convertase (C3bBbC3b). This Pathway can be initiated in three distinct ways:

- **Tick-over Pathway**

  In this it utilizes the four serum components C3, Factor B, Factor D and properdin.

- **Initiated by the protein**

  Properdin is utilized in this process.
- **Other initiated by proteases such as Thrombin and kallikrein.**

ALTERNATIVE TICKOVER PATHWAY

The pathway is initiated when C3, is at high concentration in serum. It undergoes spontaneous hydrolysis at its internal thioester bond, yielding the molecule C3(H_2O).

INITIATION

C3(H_2O) accounts for approximately 0.5% of plasma C3 and in the presence of serum Mg^{2+} binds another serum protein, Factor B. when bound to C3(H_2O), Factor B becomes susceptible to cleavage by serum protease, Factor D. Factor D then cleaves Factor B into two fragments Ba (smaller subunit) and Bb (it is a catalytically active subunit and remains bound to C3(H_2O). This C3(H_2O)Bb complex, at this point is still in the fluid phase (in the plasma, not bound to the cells). It has C3 convertase activity as it rapidly cleaves many molecules of C3 into C3a and C3b. This initiating C3 convertase is constantly being formed in plasma and breaking down a few C3 molecule, but it is then just as rapidly degraded. If there is an infection present, the newly formed C3b molecules bind nearby microbial surfaces via their thioester linkages. Since Factor B is capable of binding to C3b as well as to C3(H_2O), Factor B now binds the newly attached C3b molecules on the microbial cell surface and thus, again becomes susceptible to cleavage by Factor D. C3bBb complex is formed, it is located on the microbial membrane surface (like C4b2b complex in classical pathway).

AMPLIFICATION

C3bBb has C3 convertase activity and at this point it changes from fluid phase to predominant C3 convertase. C3bBb is unstable unless bound by properdin (protein from the serum). Once it gets stabilized by properdin, these cell-associated C3 convertase complex rapidly generate large amounts of C3b at the microbial surface. This in turn results in the binding of more factor B, resulting its cleavage and activation and resulting in

amplification of the rate of C3b generation. This pathway is rapid and once the pathway is initiated, more than 2×10^6 molecules of C3b is deposited on a microbial surface in less than 5 min.

TERMINATION

C5 convertase is formed by addition of C3b to the C3 convertase complex in the alternative complement system. C3 convertase is also stabilized by binding to Factor P. C5 convertase can be denoted by C3bBbC3b. C5 then further goes on to form the MAC (Membrane Attack Complex) by activating various components like C6, C7, C8 and C9.

ALTERNATIVE PROPERDIN-ACTIVATED PATHWAY

Properdin is a regulatory factor that stabilizes C3bBb, membrane bound C3 convertase. Recent study show that, properdin may also serve to initiate it. When properdin molecules are attached to an artificial surface and allowed to interact with purified complement components in the presence of Mg^{2+}, the immobilized properdin bound C3b and Factor B. This bound Factor B is proved to be susceptible to cleavage by Factor D. Thus, resulting in the formation of effective C3 convertase. Properdin also acts as a pattern recognition receptor and provides greater selectivity as compared to Tickover pathway. This pathway relies on the preexistence of low levels of C3b.

ALTERNATIVE PROTEASE ACTIVATED PATHWAY

The complement and blood coagulation pathways both use protease cleavage and conformational alterations to modify enzyme activities. Recently, it has been demonstrated that initiation of the coagulation cascade may result in the cleavage of physiologically relevant amounts of C3 and C5 to produce C3a and C5a.

Thrombin was seen to be capable of cleaving C5 and release of active C5a in an immune-complex model of acute lung inflammation. Plasmin are also capable of generating both C3a and C5a. When blood platelets are activated during a clotting reaction, they release high concentration of ATP and Ca^{2+} along with serine/threonine kinases. These enzymes act to phosphorylate extracellular proteins including C3b, as C3b is less susceptible to proteolytic degradation than its unphosphorylated form.

All pathways converge at the formation of the C5 convertase. The binding of the C5b, C6 and C7 leads to the conformational change that exposes hydrophobic regions on the surface of the C7 components capable of inserting into the interior of the microbial membrane. The reaction however occurs on an immune complex or other non-cellular activating surface, then the hydrophobic binding sites cannot anchor the complex and it is released. The released C5b67 complexes can insert into the membrane of nearby cells and mediate "innocent bystander" lysis. C8 is made of two peptide chains C8β and C8αγ. Binding of C8β and C5b67 induces a conformational change in the C8 dimer such that the hydrophobic domain of C8αγ can insert into the interior of the phospholipid membrane. The C5b678 complex creates a small pore, 10Å in diameter and formation of this pore can lead to lysis of red blood cells but not of nucleated cells. Final step is the formation of the MAC is binding and polymerization of C9 to the C5b678complex. 10 to 19 molecules of C9 can be bound and polymerized by a single C5b678 complex. C9 molecules undergo transition, so that they too can insert into the membrane. The completed MAC has a diameter of 70Å to 100Å. Loss of the plasma membrane integrity leads irreversibly to cell death.

1.4 DIVERSE FUNCTIONS OF COMPLEMENT

Complement has important function in innate immunity. Some complement receptors play an important role in regulating complement activity by mediating proteolysis of biologically active complement components. Anaphylatoxins also show increase in the number of complement receptors by as much as tenfold. There are various different receptors that play a major role in the complement system.

1. CR1 (CD35)

CR1 receptor is expressed on both leukocytes and erythrocytes. It binds with high affinity to C4b, C3b and smaller C3b breakdown products. CR1 receptors on erythrocytes bind immune complexes and take them to liver where they are picked up by phagocytes and cleared. Binding of complement-opsonized microbial cells via CR1 on phagocytes results

in receptor-mediated phagocytosis and the secretion of proinflammatory molecules such as IL-1 and prostaglandins. CR1 on B-cells mediates antigen uptake of C3b-bound antigen, leading to its degradation in B-cell lysosomal system and subsequent presentation to T cells. This makes CR1 have a role in both adaptive and innate immune response. It also mediates the protection of host cells against the ruins of the complement attack. Generally, it happens as it serves as a cofactor for the destruction of C3b and C4b by cleavage on the host cell membranes by Factor I. CD35 also acts as an accelerator of the decay of the C3 and C5 convertases. C3b either in solution or bound to the surface of cells, is subjected to breakdown by endogenous proteases.

2. CR2 (CD21)

CR2 are expressed on B cells and binds specifically to the breakdown products of C3b: iC3b, C3d and C3dg. C3b can form covalent bonds with antigens. Presence of CD21 on B-cells enables the B cell to bind antigen via both the B cell receptor and CD21. This ability to simultaneously co-engage antigen through two receptors has the effect of reducing the antigen concentration necessary for B-cell activation by up to a hundred fold.

3. CR3

CR3 is a complex of CD11b and CD18.

4. CR4

CR4 is a combination of CD11c and CD18. They are important in the phagocytosis of complement-coated antigens. CR4 binds to C3b and several of its breakdown products, including iC3b, C3c and C3dg.

5. CRIg

CRIg also binds to C3b. These receptors are expressed on macrophages resident in the fixed tissues, including on the kupffer cells of the liver. The major significance of these receptors is in clearing C3b-opsonized antigen by facilitating their removal from circulation in the liver. Animals which are deficient are therefore subjected to higher mortalities during infections.

6. C3aR, C5aR and C5L2

They are the members of the G protein coupled receptor (GPCR). C3aR and C5aR mediate inflammatory functions after binding the small anaphylatoxins, C3a and C5a respectively. Whereas C5L2 receptor binds C5a but is not functionally coupled to the G protein signaling pathway as used by C5aR.C5aR and C5L2 are structurally similar and is expressed on some of the same cells. C5L2 appears to modulate C5a signaling through C5aR and C5L2 knockout animals express enhanced inflammatory response upon C5a binding to its receptor.

1.5 REGULATION OF THE COMPLEMENT SYSTEM

Regulation of the system helps us to avoid any damage caused to the healthy host tissue. The complement proteins should be maintained in such a way that when they are not required the destructive potential of complement proteins is confined to the appropriate pathogen surfaces. There are different ways by which the host protects itself:

1) Complement Activity is passively regulated by protein stability and cell-surface composition.

   The protection of vertebrate host cells against complement mediated damage is achieved by general, **Passive regulatory mechanism** and specific**, Active regulatory mechanism**. There is a term known as relative instability which is related to many complement components. It refers to the first means by which the host protects itself. For example: C3 convertase of the alternative pathway (C3bBbC3b). This C3 convertase has a half-life of only 5 min. So, the attachment of properdin in this case helps to stabilize the complex. **The passive regulatory mechanism depends upon the difference in the cell surface carbohydrate composition of host versus microbial cells**. This mechanism is effectively shown by some fluid phase proteases that destroy C3b, bind much more effectively to the host cell, which bear high levels of sialic acid. Whereas, in the case of active

regulatory mechanism a series of active regulatory proteins act to inhibit, destroy, or tune down the activity of complement proteins and their active fragments.

2) The C1 inhibitor, C1INH, promotes dissociation of C1 components.

C1INH

C1INH is a C1 inhibitor i.e. it doesn't allow the formation of the C1 complex which is further unable to form C3 convertase. It belongs to the class of serine protease inhibitors (Serpins). It is a plasma protein that binds to the active site of serine proteases, effectively poisoning them. Mechanism of action involves forming a complex with the C1 proteases which causes the C1r2s2 to dissociate from C1q and prevents further activation of C4 or C2. The C1INH is only plasma protein capable of inhibiting both classical and lectin pathway as it inhibits both C3b and the serine protease MASP2.

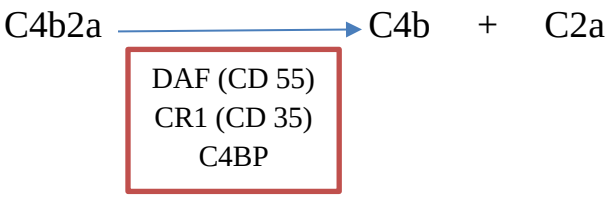
3) Decay Accelerating Factor (DAF) promotes decay of C3 convertases.

In complement activation the reaction catalyzed by C3 convertase enzyme is a major amplification step. DAF [CD 55], CR1 and C4BP (C4 binding protein) accelerate the decay of the C4b2a. The enzymatically active C2a diffuses away, and the residual membrane-bound C4b is degraded by another regulatory protein; Factor I. In Alternative Pathway, DAF and CR1 are used and joined by Factor H. Factor H separates the C3b components of the alternative pathway from Bb which together form the C3 convertase. C3b gets degraded and Bb diffuses away.

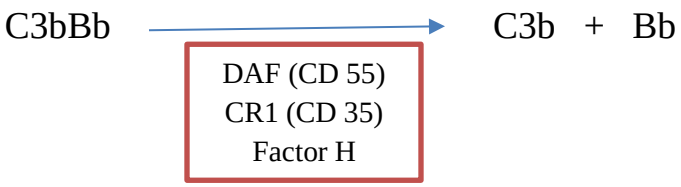
DAF and CR1

DAF and CR1 are membrane bound components, their expression is restricted to the host cell. Factor H which binds these two is a soluble regulatory complement component. Factor H has host specific function. It is generally seen by its binding to polyanions such as sialic acid and heparin essential components of eukaryotic cell surfaces. C4BP is also soluble regulatory complement component. It is bound by host cell membrane proteoglycans such as heparin sulfate. Thus, protects from deposition of complement components.

In Classical Pathway



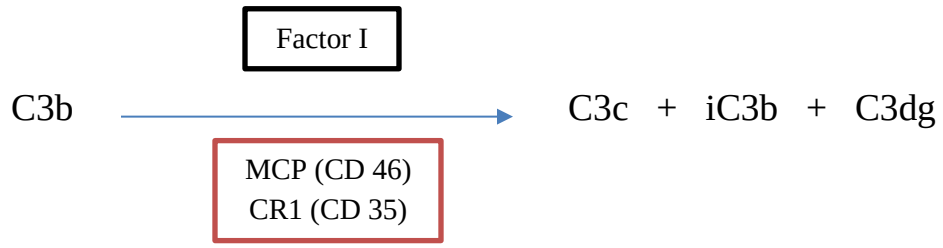
In Alternative Pathway

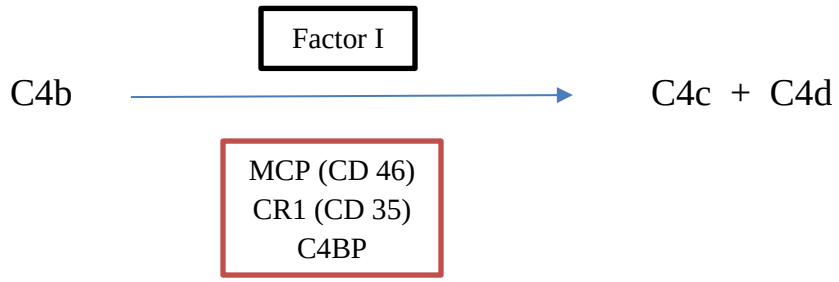


4) Factor I degrades C3b and C4b.

Factor I involved here is a soluble and constitutively active serine protease. It has the ability to cleave membrane associated C3b and C4b into inactive fragments. The presence of its cofactors is necessary for its proper function, which are Membrane Cofactor of Proteolysis (MCP) and CR1. They are found on the host

cell and not on microbial cells thus, invading microbes can't be destroyed. Factor H, C4BP, and CR1 also work in this pathway. Factor H and C4BP bind to host cell surface peptidoglycans. So, cleavage of membrane-bound C3b on host cell is done by Factor I in collaboration with CD 46, CR1 and Factor H. In case of C4b it's affected by Factor I, but this time in collaboration with CD 46, CR1 and soluble cofactor C4BP. CD 46 is implicated as a factor in the control of apoptosis of dying T-cells. These following equations explains the role of Factor I:

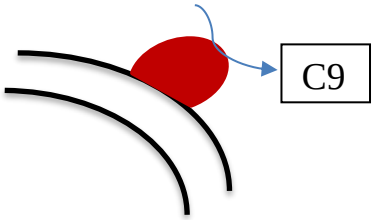




5) Protectin inhibits the MAC attack

The inappropriate assembly of MAC complexes on healthy host cells is observed due to the robust Antibody response or of an inflammatory response accompanied by extensive complement activation**. Protectin (CD 59) is a host cell surface protein which is attached by phosphatidylinositol anchor.** CD 59 binds to any C5b678 complexes that may be deposited on host cells and prevents their insertion into the host cell membrane. Protectin also blocks further C9 addition to developing

MACs. **Vitronectin** is another soluble complement serine protein which binds to the fluid phase C5b67 complexes released from microbial cells, preventing their insertion into the host cell membranes.



C5b, C6, C7, C8 → Protectin (CD 59) Vitronectin

MAC inhibition prevents C9 binding and polymerization

6) Carboxypeptidases can inactivate the anaphylatoxins C3a and C5a.

This process regulates the complement system by cleaving of the C-terminal arginine residues from both C3a and C5a by serum carboxypeptidases. Serum carboxypeptidases causes rapid inactivation of anaphylatoxin activity. It's a general class of enzyme that removes amino acids from the carboxyl termini of proteins. They remove arginine residues from carboxyl termini of C3a and C5a to form so called des-Arg ("without Arginine"). Specific enzymes in the case of anaphylatoxin are carboxypeptidases N, B and R.

C3a → C3a des Arg

C5a → C5a des Arg

Carboxypeptidase N
Carboxypeptidase B
Carboxypeptidase R

1.6 DYSREGULATION OF THE COMPLEMENT SYSTEM

In normal condition the RBC doesn't undergo these pathway. But sometimes due to the absence of some protein or other mutation it leads to the activation of the complement pathways thus, leading to the autoimmune diseases shown in figure 4. In such cases our body produces antibodies against our own cells as they recognize them as foreign or pathogenic. One such instance taken into consideration here is **Paroxysmal nocturnal hemoglobinuria** (**PNH**).

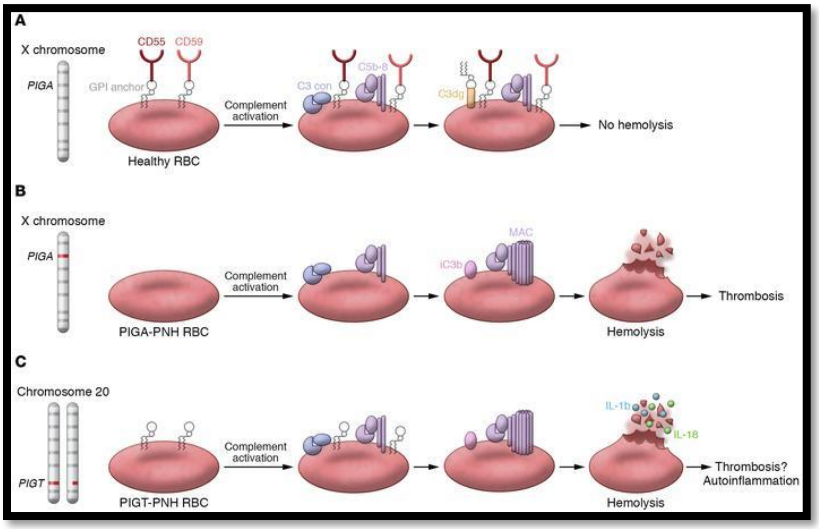


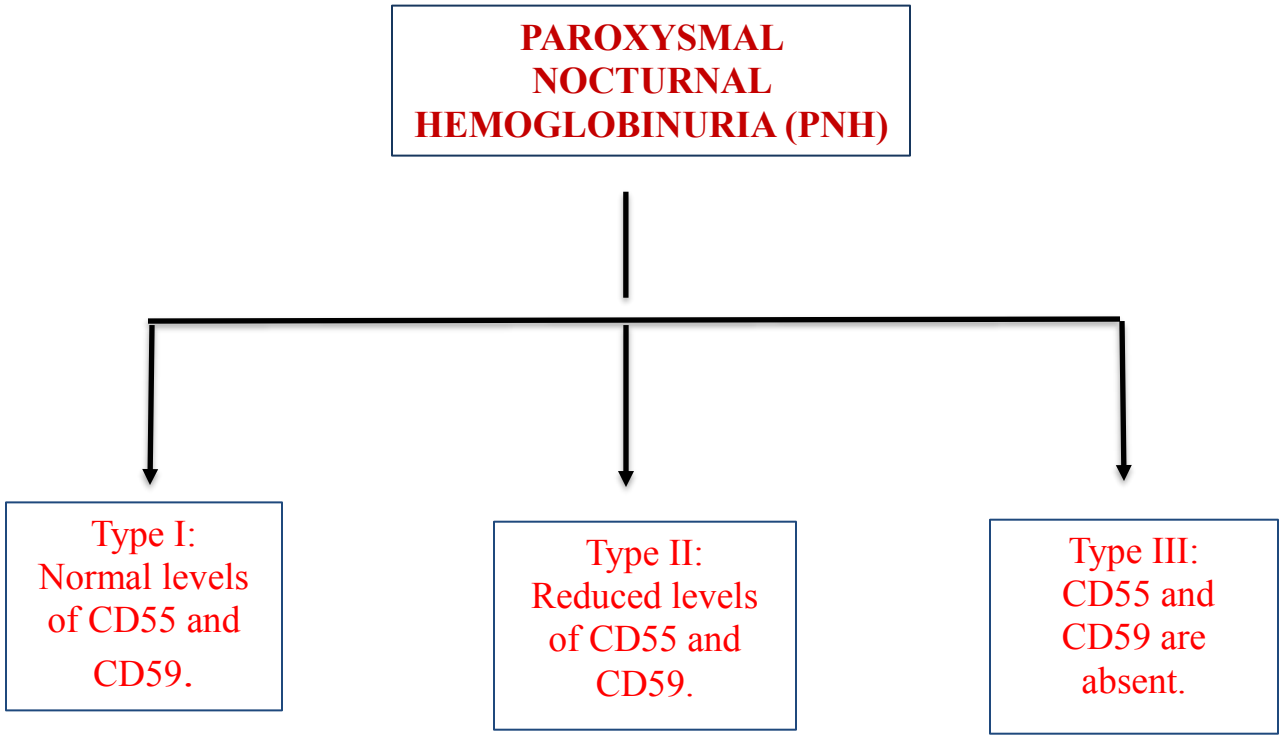
Figure 4: Dysregulation of the Complement system

(https://doi.org/10.1172/JCI131647)

It is the only hemolytic anemia caused by an acquired intrinsic defect in the cell membrane. PNH occurs when mutations of a gene called PIG-A occur in a bone marrow stem cell. Stem cells give rise to all the mature blood elements including red blood cells, which carry oxygen to our tissues; white blood cells, which fight infection; and platelets, which are involved in forming blood clots. PIG-A gene codes for a protein required for synthesis of **N-acetylglucosaminyl phosphotidylinositol**, which is the first intermediate in biosynthetic pathway of Glycosylphosphatidylinositol (GPI) anchor (*Risitano et.al.,2013*). In PNH, the affected stem cell passes the PIG-A mutation to all cells derived from the abnormal stem cell. Cells harboring PIG-A mutations are deficient in a class of proteins

called GPI-anchored proteins. Certain GPI-anchored proteins protect red blood cells from destruction, some are involved in blood clotting, and others are involved in fighting infection and maintaining the cellular integrity of the cell. The majority of PNH-related issues, including destruction of red blood cells (hemolytic anemia), blood clots (thrombosis), and infection, result from a deficiency of these proteins. In some cases of PNH it is observed that it develops on its own and is referred to as "primary PNH" and the other is in the context of bone marrow disorders such as aplastic anemia also referred as "secondary PNH". A minority of affected people have the telltale red urine in the morning. This red coloration of the urine is due to the presence of hemoglobin and hemosiderin from the breakdown of red blood cells. The Symptoms of PNH are significant fatigue or weakness, bruising and bleeding easily, blood clots, appearance of small red dots on the skin that indicates bleeding under the skin.

CLASSIFICATION OF ERYTHROCYTES IN PNH CELLS (*Parker et.al., 1982*)



Rationale of Study

2. RATIONALE OF STUDY

Blood is the important component of our body as it performs a lot of functions. Some immune disorders are related to blood like Common Blood-Related Autoimmune Disorders, Lupus, Vasculitis, Autoimmune hemolytic anemia. There is no cure for such diseases except only for bone marrow transplant which is successful in case of only a few patients. Lysis of the cells caused in these disorders are mostly caused by damage or mutation in the complementary system. Overlook of all the pathways in the complement system will provide us a better knowledge of the disease and an extensive study can be carried out. These pathways can then be used as the target in case of targeted therapy and help us cure various autoimmune and other complement pathway related disorders. The role of these regulatory proteins need to be explored as to see how these disease condition can be overcome to prevent RBC lysis. Thus, this study will provide us a better understanding of the complement regulatory proteins and their link to the different complement pathway.

Objective

3. OBJECTIVE

To observe the effect of complement regulatory proteins in the process of RBC lysis.

Materials and Methods

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Alsever Buffer

Alsever's buffer is a liquid used as a suspension medium and stabilizer for red blood cells (also called erythrocytes). It is used as the blood cell preservative that permits the storage of blood cells at refrigerator temperatures for approximately 10 weeks (*Archives et.al.,2018*). The composition of Alsever buffer is given in Table 1.

Table 1: Composition of Alsever Buffer

Components	Concentration (%)
Dextrose	2.05
Sodium Citrate	0.8
Citric Acid	0.055
Sodium Chloride	0.42

4.1.2 Gelatin veronal Buffer (GVB)

Gelatin Veronal Buffer has both the presence of calcium and magnesium ions which helps in the activation of both classical and alternative pathway. Table 2 explains about the complete composition of GVB buffer.

Table 2 : Composition of Veronal Buffer

Components	Concentration (mM)
$CaCl_2$	0.15
Nacl	141
Sodium Barbital	1.8

Barbituric acid	3.1

Gelatin Veronal Buffer (GVB) consists of Veronal buffer (1X), 0.5mM $MgCl_2$ and 0.1% gelatin. The pH of the buffer is maintained at 7.3 – 7.4.

4.1.3 EGTA

EGTA stands for Ethylene glycol-bis (2-aminoethyl ether) tetraacetic acid.
It has a very important role as it is a chelating agent and has high affinity for Ca^{2+} ions but lower affinity for Mg^{2+} ions. It is mostly used as a buffer to resemble the pH of a living cell. EGTA of 10 mM concentration is used in this experiment.

4.1.4 Antibodies

These are the Antibodies used against the regulatory protein CD 55 (Decay Acceleration Factor), CD 59 (Protectin) and C3 component of the complement system. The Anti C3b Antibody used here is PE conjugated which serves as the detector for the target of interest. PE is maximally excited at 565 nm and emits at 573 nm, which is in the yellow-orange region of the visible spectrum

Table 3 : Antibodies used

Sr. no.	Name of the Antibody used	Host Isotype	Maker	Quantity used
1.	Anti-CD59 Monoclonal Antibody	Mouse IgG2a	Invitrogen (Waltham, USA)	10 µg/ml
2.	Anti-CD55 Monoclonal Antibody	Mouse IgG1	Merck (New Jersey, USA)	10 µg/ml
3.	Anti-human C3/C3b/iC3b Monoclonal Antibody	Mouse IgG1	Cedarlane (Surrey, Canada)	0.1 µg/10^6 cells

4.1.5 Normal Human Serum (NHS)

4.1.6 1X PBS

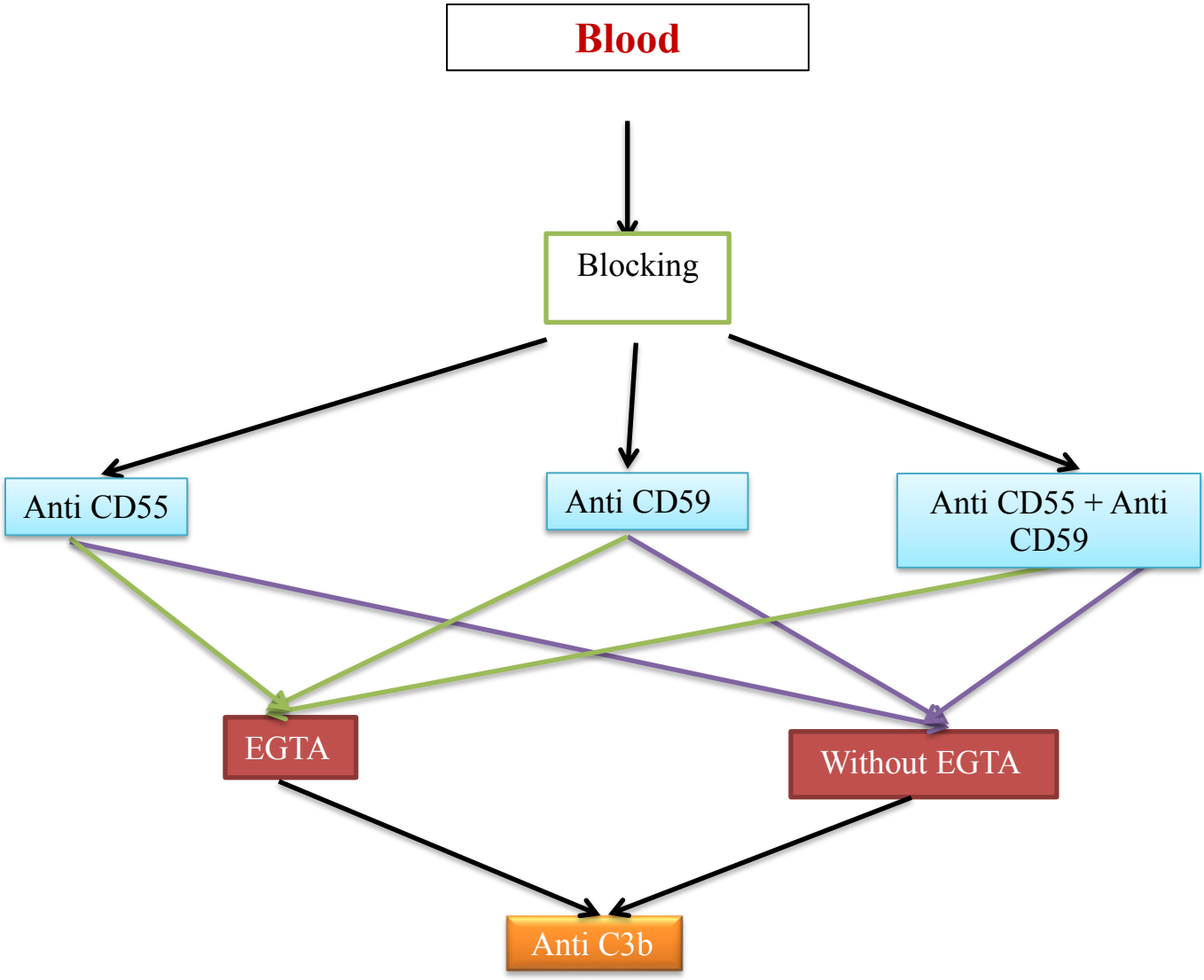
PBS (Phosphate Buffered Saline) is used to wash off the RBCs so any other contamination can be avoided and only RBCs are left behind. The elements of 1X PBS is listed in Table 4.

Table 4 : Composition of 1X PBS:

Components	Concentration (mM)
KCl	2.7
Nacl	137
Na_2HPO_4	10
KH_2PO_4	1.8

The pH of the buffer is maintained at 7.4.

4.2 Simulation of PNH condition in the blood cells



EGTA – Ethylene glycol-bis (2-aminoethyl ether) tetraacetic acid.

Role: Chelating agent and has high affinity for Ca^{2+} ions but lower affinity for Mg^{2+} ions. It is mostly used as a buffer to resemble the pH of a living cell.

Simulation of the PNH conditions helps us to have a better understanding of the disease condition and its types.

4.3 Methods

4.3.1 Standardization Assay

- Standardization is the most important aspect in any bioassay as it provides us with values that can be later on compared with the samples in order to check the desired results.
- In this assay antibodies against CD 55 and CD 59 were used. This binding allows us to monitor the binding of the antibodies to their specific positions. Later, the use of these antibodies will help us to create the disease condition that is PNH condition *in vitro*.

4.3.2 C3b deposition assay

- C3b deposition is recorded in this assay as C3 is an important component in the complement pathway. C3b which is obtained after the hydrolysis of the C3 component with the help of an enzyme C3 convertase. It is a crucial intermediate in the complement process.
- Anti-C3b antibody is used in the assay which basically binds to the C3, iC3b and C3b. These are the fragments of the C3 component formed when C3 undergoes hydrolysis or gets degraded. This deposition of C3 and other components is further analyzed in the Fluorescence-activated cell sorting (FACS).

GVB is added and centrifuged at 2000 rpm for 5 min (this step is repeated 3-4 times).

Cells are diluted 1:200 times (3980 µl GVB without EGTA and 20µl RBC). 10×10^6 cells are seeded per well and then allowed to settle down at 2000 rpm for 3 min. Cells are again given a wash with their respective buffers (with EGTA and without EGTA)

Collected blood is centrifuged at 2000 rpm for 5 min and then supernatant is removed.

100µl of Antibodies (Anti CD-55 and Anti CD-59) is added to the respective wells and incubated at room temperature for 30 min.

4.3.3 Lysis assay

- Complement system is involved in the lysis of the RBCs. Lysis in RBCs happens when any ingredient of the complement system is mutated or absent.
- Due to this lysis of the RBCs a color difference is observed in the plate compared from the wells in which RBCs have not undergone lysis.
- The readings are then taken in the ELISA plate reader at 405 nm.

Results

And

Discussion

5. RESULTS AND DISCUSSION

5.1 Standardization assay

As we are using the blood which is made of various components and the main focus here is on the RBCs. Standardization of the assay allows us to reduce the risk and contamination of our samples later by the other components. Thus, the RBCs are incubated in various combinations such as: NHS, NHS and Anti C3b and just Anti C3b. They were incubated in two kinds of conditions one with EGTA buffer and without EGTA buffer.

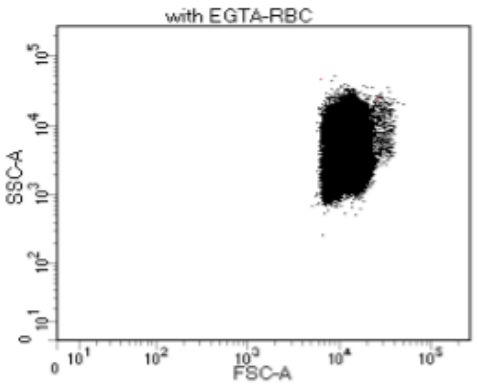
Table 5 : Controls used in the Standardization Assay

Sr. no.:	Controls
1.	RBC
2.	RBC + NHS
3.	RBC + NHS + Anti C3b
4.	RBC + Anti C3b

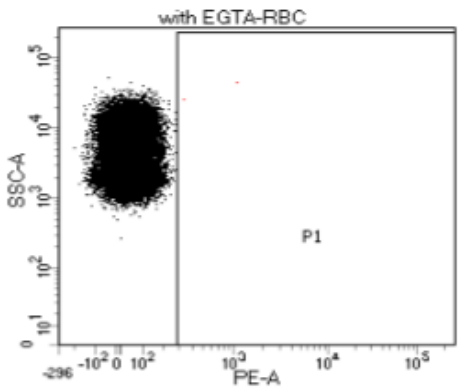
FACS RESULTS

FACS provides us with the C3b deposition data which is an important component in the complement system. Upon determining the C3b deposition we can determine the RBC lysis status. PE conjugated antibody is used here which binds to C3b and fragments of C3b. Following graphs depict the FACS results of the controls.

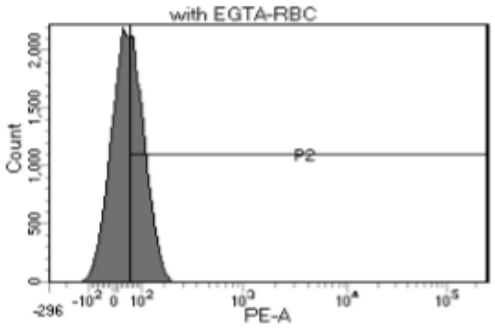
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC
Record Date:	Jan 30, 2020 3:55:14 PM
$OP:	Administrator
GUID:	693b4abe-83e5-4af3-bd6f-7782...

Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,194	34
P1	2	0.0	35,621	683
P2	19,360	38.7	5,281	91

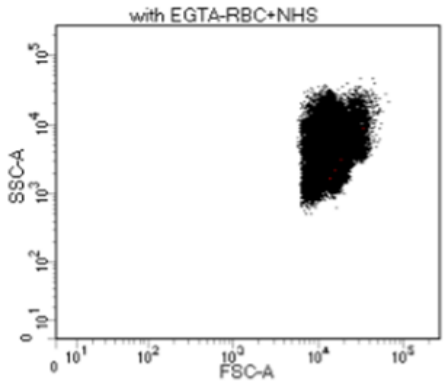
Figure 5 : Control 1- RBC

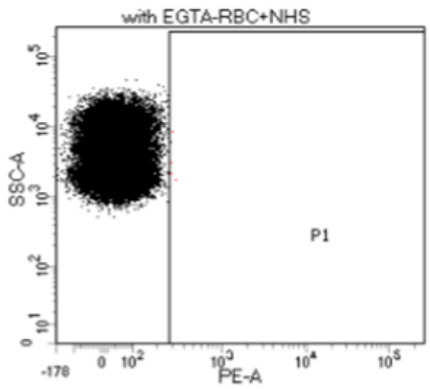
It represents the FACS results of the control 1 which includes only RBCs with EGTA buffer..

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. Figure C provides us with the histogram depiction and the stable peak.

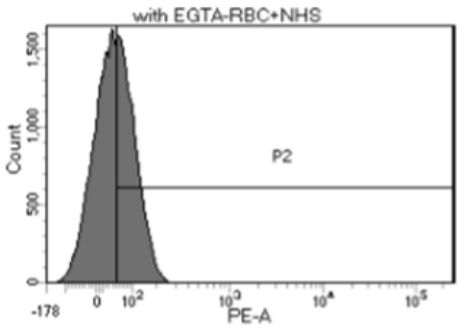
A)

B)





C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+NHS
Record Date:	Jan 30, 2020 3:56:02 PM
$OP:	Administrator
GUID:	ca4eccde-01fd-4bfe-bc2e-2b01...

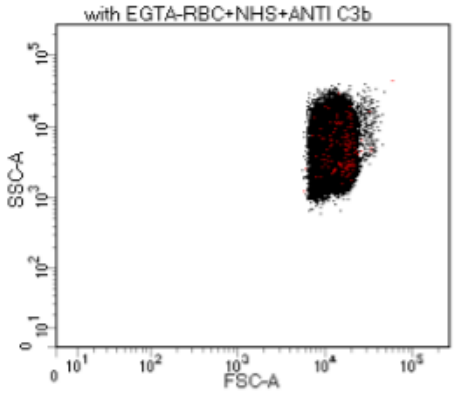
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,336	36
P1	4	0.0	3,970	296
P2	20,094	40.2	5,509	92

Figure 6 : Control 2- RBC + NHS

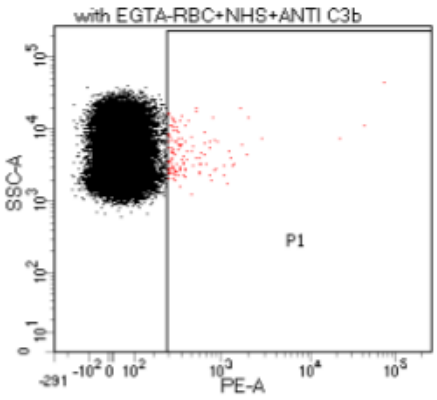
It represents the FACS results of the control 2 which includes RBC + NHS with EGTA buffer.

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. Figure C provides us with the histogram depiction and the stable peak.

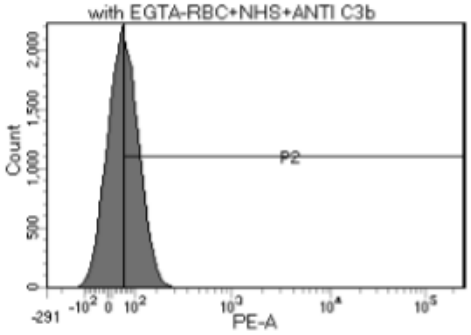
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+NHS+ANTI C3b
Record Date:	Jan 30, 2020 3:57:12 PM
$OP:	Administrator
GUID:	ce196cbf-d490-4a63-91e6-b09...

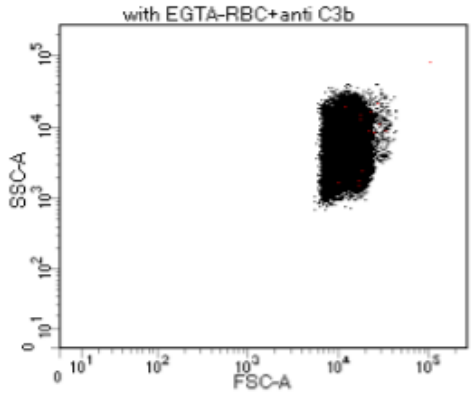
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,575	44
P1	129	0.3	6,507	1,595
P2	21,681	43.4	5,639	104

Figure 7 : Control 3 - RBC + NHS + Anti C3b

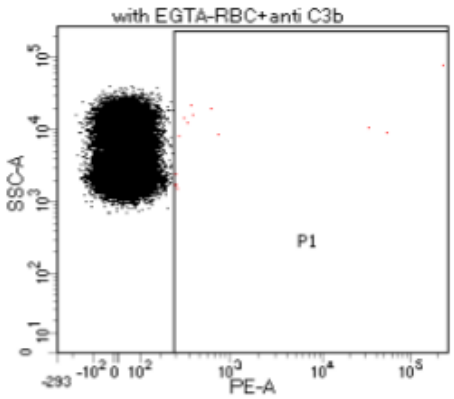
The FACS results of the control 3 includes RBC + NHS + Anti C3b with EGTA buffer.

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. As in this case 0.3% of deposition is observed which is very less and hence considered negligible. Figure C provides us with the histogram depiction and the stable peak.

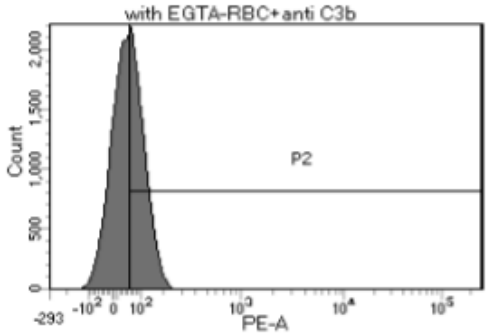
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+anti C3b
Record Date:	Jan 30, 2020 3:58:45 PM
$OP:	Administrator
GUID:	32ba3f51-480c-4bd4-aaf4-780e...

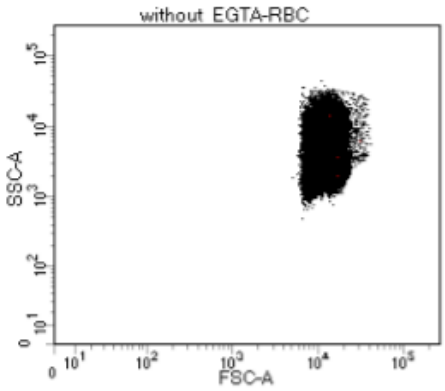
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,818	42
P1	14	0.0	15,055	23,281
P2	20,213	40.4	5,893	107

Figure 8 : Control 4 - RBC + Anti C3b

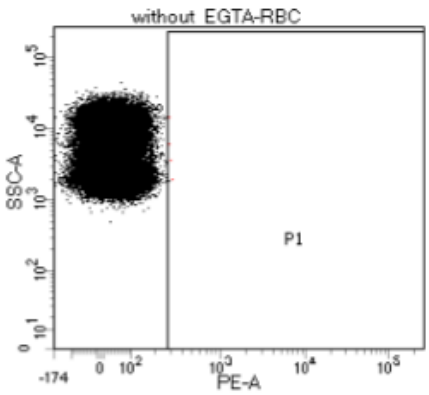
The FACS results of the control 4 includes RBC + Anti C3b with EGTA buffer.

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. No C3b deposition is observed . Figure C provides us with the histogram depiction and the stable peak.

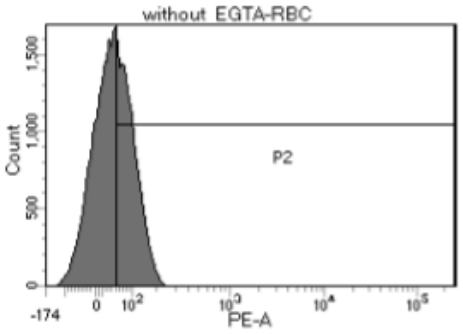
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC
Record Date:	Jan 30, 2020 4:25:03 PM
$OP:	Administrator
GUID:	9222727e-7615-4eef-84d5-c9e...

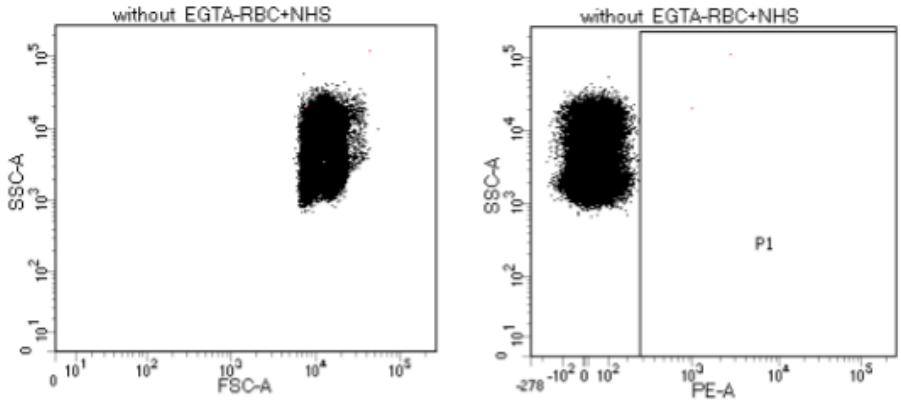
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,710	34
P1	4	0.0	6,559	290
P2	19,431	38.9	5,816	91

Figure 9 : Control 1- RBC

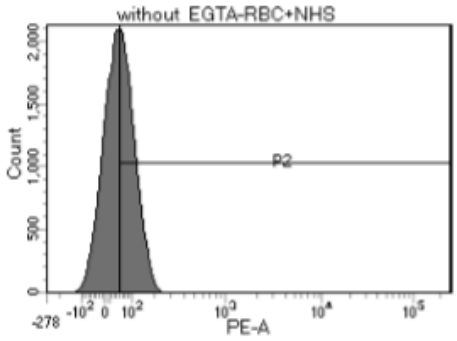
It represents the FACS results of the control 1 which includes only RBCs in without EGTA buffer..

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. Figure C provides us with the histogram depiction and the stable peak.

A) B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+NHS
Record Date:	Jan 30, 2020 4:25:55 PM
$OP:	Administrator
GUID:	bdd5136a-5d90-4184-b36e-d9...

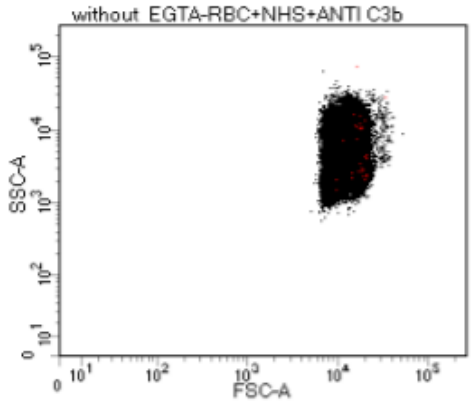
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,980	35
P1	2	0.0	68,857	1,779
P2	19,941	39.9	6,060	91

Figure 10 : Control 2- RBC + NHS

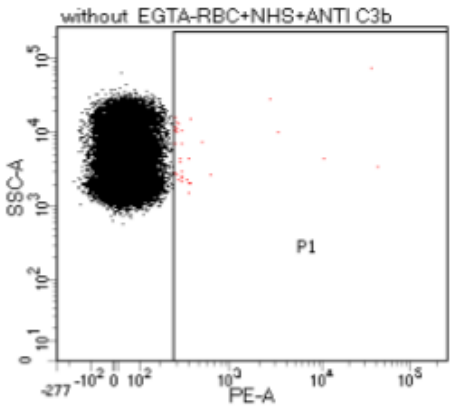
It represents the FACS results of the control 2 which includes RBC + NHS in without EGTA buffer..

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. Figure C provides us with the histogram depiction and the stable peak.

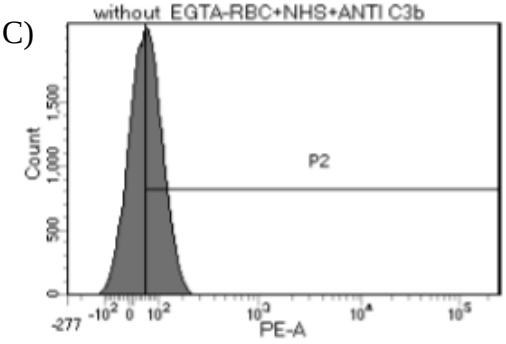
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+NHS+ANTI C3b
Record Date:	Jan 30, 2020 4:27:01 PM
$OP:	Administrator
GUID:	d4c399ec-8567-495c-af22-b73...

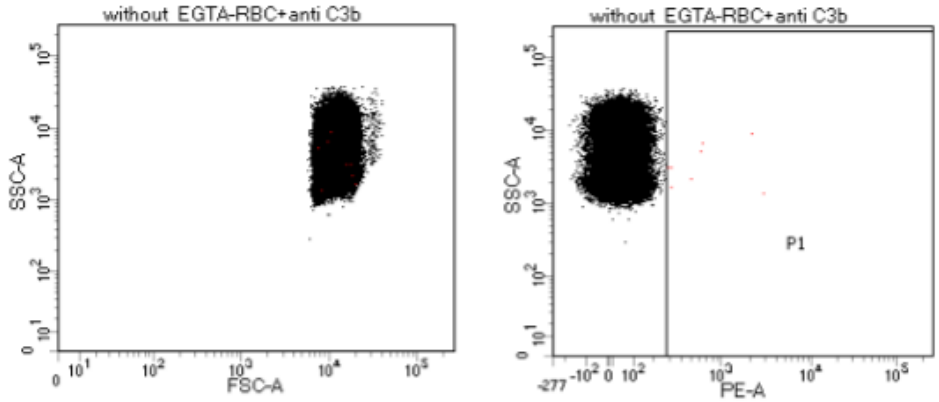
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,815	42
P1	33	0.1	9,081	3,161
P2	21,346	42.7	5,869	98

Figure 11 : Control 3- RBC + NHS + Anti C3b

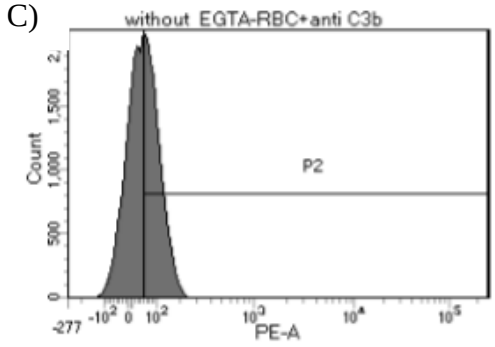
The FACS results of the control 3 includes RBC + NHS + Anti C3b in without EGTA buffer..

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition.0.1% of deposition is seen which is almost negligible. Figure C provides us with the histogram depiction and the stable peak.

A) **B)**



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+anti C3b
Record Date:	Jan 30, 2020 4:28:08 PM
$OP:	Administrator
GUID:	28538eef-4337-49f3-ad93-293a...

Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,716	35
P1	8	0.0	4,026	939
P2	19,758	39.5	5,729	91

Figure 12 : Control 4- RBC + Anti C3b

The FACS results of the control 4 includes RBC + Anti C3b in without EGTA buffer..

Here in the figure A the black spot obtained represents the presence of granulocyte which are RBCs. Figure B depicts the gates that are created in order to check the C3b deposition. No deposition is seen. Figure C provides us with the histogram depiction and the stable peak.

The above controls were performed in both EGTA and without EGTA buffer as to check error and standardize the protocol before proceeding towards the samples. From the above results it has been concluded that there is no C3b deposition seen in our controls. In a few controls where it is observed it is so minor that it can be neglected. It may also be due to some other molecules. Thus, it can be said that our controls are acceptable and considerate.

5.2 C3b deposition assay

As seen C3 is the important component of the complement system. C3 convertase in the complement pathways is the adjoining point in the pathways which later converts C3 component to C3a and C3b. The C3b further joins to form the C5 convertase which undergoes MAC formation and lysis. Here in the experiment Anti CD55, Anti CD59 and a combination of both is used with the Anti C3b Antibody. Thus, creating the PNH conditions. Deposition of the C3b in the cells is then observed.

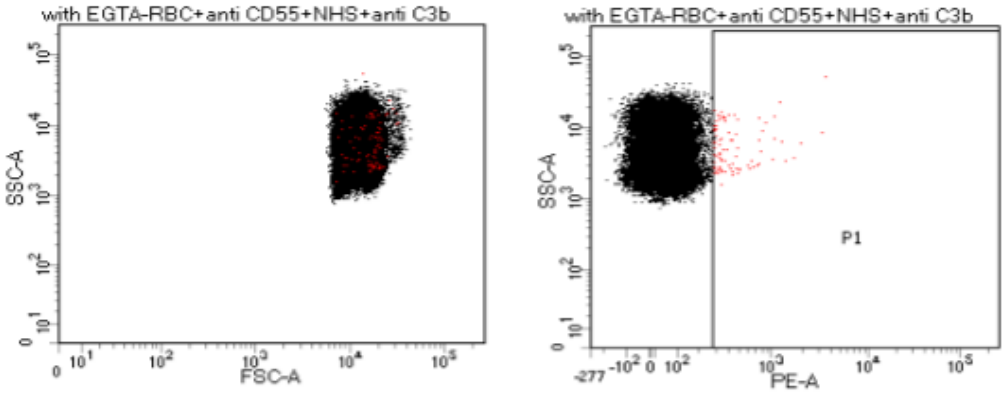
Table 6 : Samples used in the C3b Deposition Assay

Sr. no.:	Samples
1.	RBC + Anti CD55 + NHS + Anti C3b
2.	RBC + Anti CD59 + NHS + Anti C3b
3.	RBC + Anti CD55 + Anti CD59 + NHS + Anti C3b
4.	RBC + Anti CD55 + Anti CD59 + NHS + Anti C3b

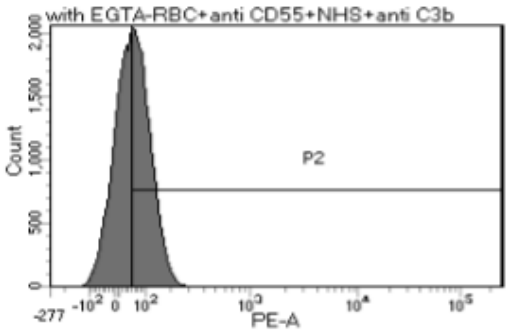
FACS RESULTS

The following figures depict the FACS results of our samples where we have stimulated the PNH conditions by blocking CD55 and CD59. In sample 1 only CD55 is blocked by Anti CD55 and in sample 2 CD59 is blocked using Anti CD59.The sample 3 and 4 are similar as the experiment was performed in duplicate just to obtain accurate results by using both Anti CD55 and Anti CD59.

A) B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+anti CD55+NHS+anti C3b
Record Date:	Jan 30, 2020 4:00:36 PM
$OP:	Administrator
GUID:	09e1effb-9ab8-4bfd-968a-61ea...

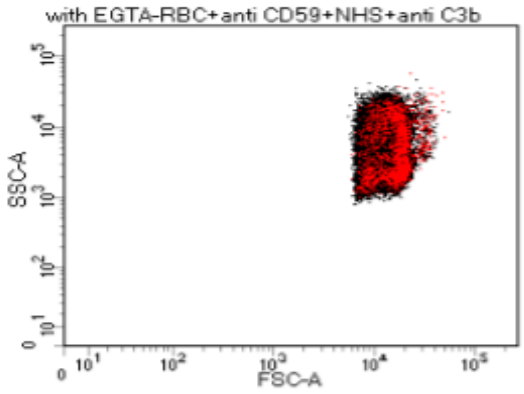
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,941	41
P1	87	0.2	7,326	547
P2	21,774	43.5	6,037	96

Figure 13 : Sample 1 - RBC + Anti CD55 + NHS + Anti C3b

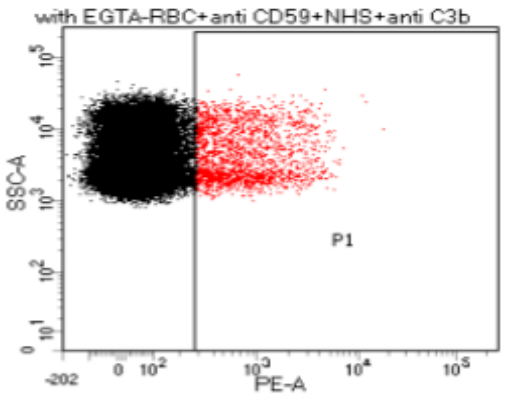
The FACS result of the Sample 1 including RBC + Anti CD55 + NHS + Anti C3b with EGTA buffer.

The figure A depicts the black spot and a few red dots obtained representing the presence of granulocyte which are RBCs. Figure B depicts the gates that are created which shows 0.2% C3b deposition. Figure C provides us with the stable histogram peak.

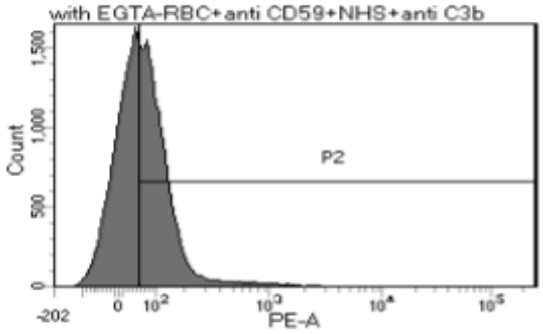
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+anti CD59+NHS+anti C3b
Record Date:	Jan 30, 2020 4:01:52 PM
SOP:	Administrator
GUID:	e0946be4-2963-4168-8924-6c5...

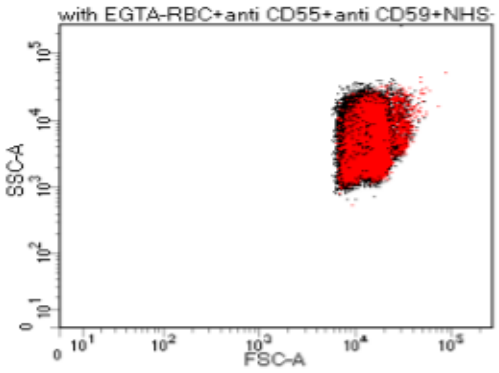
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,838	87
P1	2,093	4.2	6,436	1,013
P2	24,471	48.9	5,893	179

Figure 14 : Sample 2 - RBC + Anti CD59 + NHS + Anti C3b

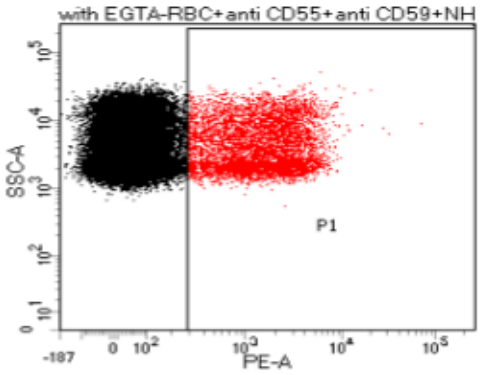
The FACS result of the Sample 2 including RBC + Anti CD59 + NHS + Anti C3b with EGTA buffer.

The figure A depicts the black spot and red dots obtained representing the presence of granulocyte which are RBCs. Red spots represent the C3b deposition. Figure B depicts the gates that are created which shows 4.2.% C3b deposition. In figure C a shift in the histogram peak is observed.

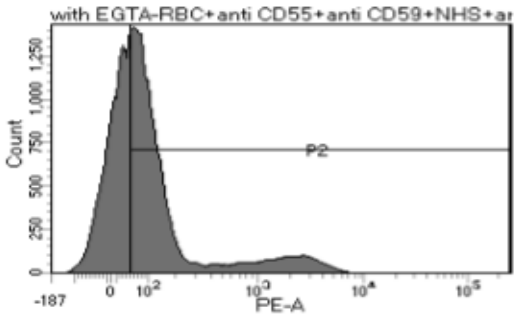
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	with EGTA
Tube Name:	RBC+anti CD55+anti CD59+NH...
Record Date:	Jan 30, 2020 4:03:13 PM
SOP:	Administrator
GUID:	7213d0cd-5d72-43ca-9059-5f9...

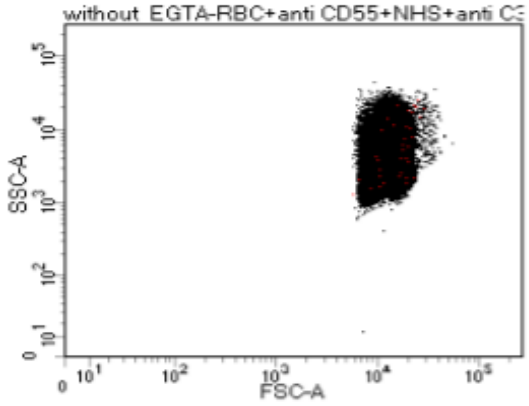
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,949	274
P1	6,186	12.4	5,945	1,828
P2	27,125	54.3	6,025	495

Figure 15 : Sample 3 and 4 - RBC + Anti CD55 + Anti CD59 + NHS + Anti C3b

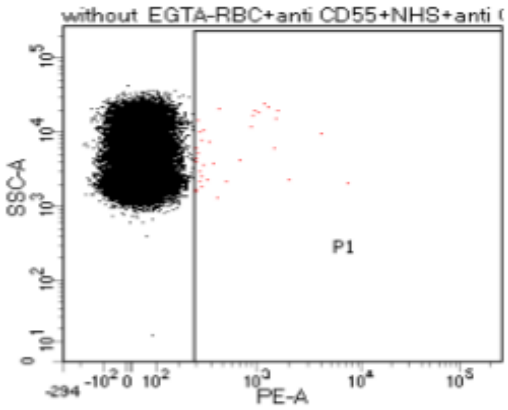
The FACS result of the Sample 3 and 4 including RBC + Anti CD59 + Anti CD59 + NHS + Anti C3b with EGTA buffer.

The figure A depicts the black spot and red dots obtained representing the presence of granulocyte which are RBCs. Red spots represent the C3b deposition. Figure B depicts the gates that are created which shows 12.4.% C3b deposition. In figure C a shift in the histogram peak is observed.

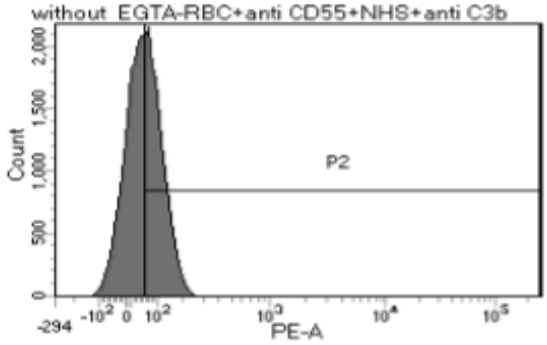
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+anti CD55+NHS+anti C3b
Record Date:	Jan 30, 2020 4:29:07 PM
$OP:	Administrator
GUID:	67ca5c26-34e3-403b-bcc8-ae1...

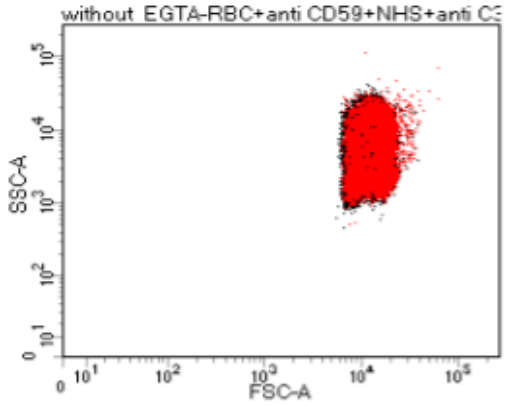
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,756	39
P1	35	0.1	8,351	907
P2	21,141	42.3	5,795	94

Figure 16 : Sample 1 - RBC + Anti CD55 + NHS + Anti C3b

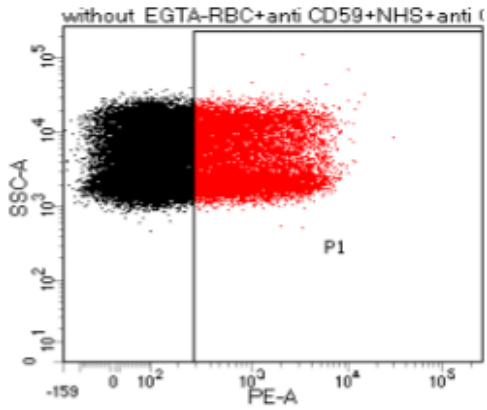
The FACS result of the Sample 1 including RBC + Anti CD55 + NHS + Anti C3b in without EGTA buffer.

The figure A depicts the black spot and red dots obtained representing the presence of granulocyte which are RBCs. Red spots represent the C3b deposition. Figure B depicts the gates that are created which shows 0.1.% C3b deposition. In figure C a shift in the histogram peak is observed.

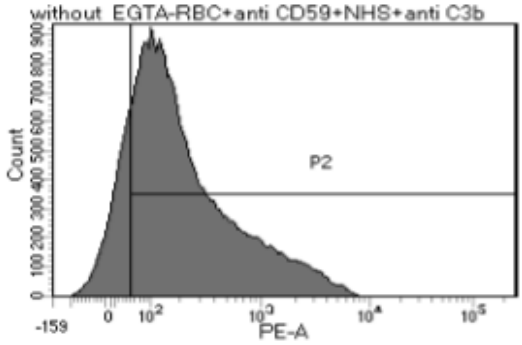
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+anti CD59+NHS+anti C3b
Record Date:	Jan 30, 2020 4:34:11 PM
$OP:	Administrator
GUID:	77893754-b401-4cd5-a287-aaa...

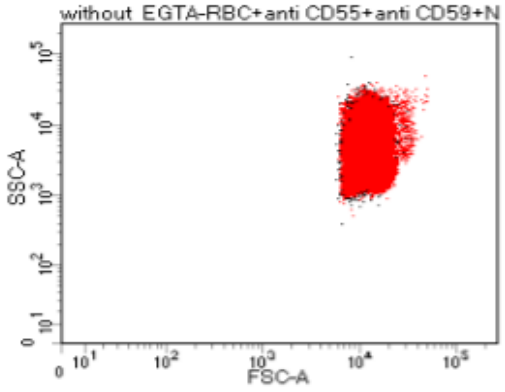
Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,779	407
P1	14,849	29.7	5,952	1,132
P2	40,714	81.4	5,805	498

Figure 17 : Sample 2 - RBC + Anti CD59 + NHS + Anti C3b

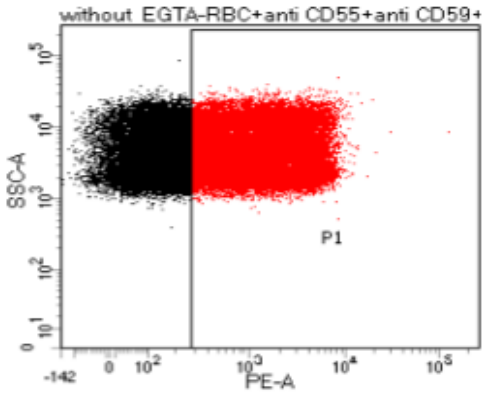
The FACS result of the Sample 2 including RBC + Anti CD59 + NHS + Anti C3b in without EGTA buffer.

The figure A depicts the black spot and red dots obtained representing the presence of granulocyte which are RBCs. Red spots represent the C3b deposition. Figure B depicts the gates that are created which shows 29.7% C3b deposition. In figure C a shift in the histogram peak is observed.

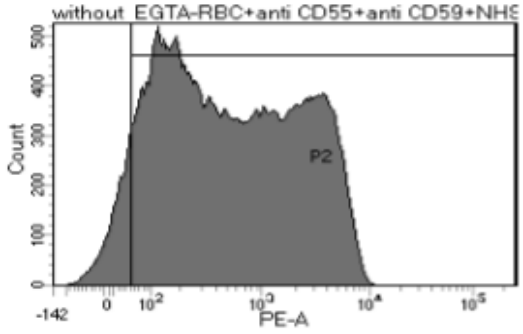
A)



B)



C)



Experiment Name:	30012020 C3b deposition with Z...
Specimen Name:	without EGTA
Tube Name:	RBC+anti CD55+anti CD59+NH...
Record Date:	Jan 30, 2020 4:36:40 PM
$OP:	Administrator
GUID:	9a58c9de-bf9e-4e31-a25e-46c...

Population	#Events	%Parent	SSC-A Mean	PE-A Mean
All Events	50,000	####	5,707	1,167
P1	29,451	58.9	5,769	1,897
P2	45,958	91.9	5,727	1,269

Figure 18 : Sample 3 and 4 - RBC + Anti CD55 + Anti CD59 + NHS + Anti C3b

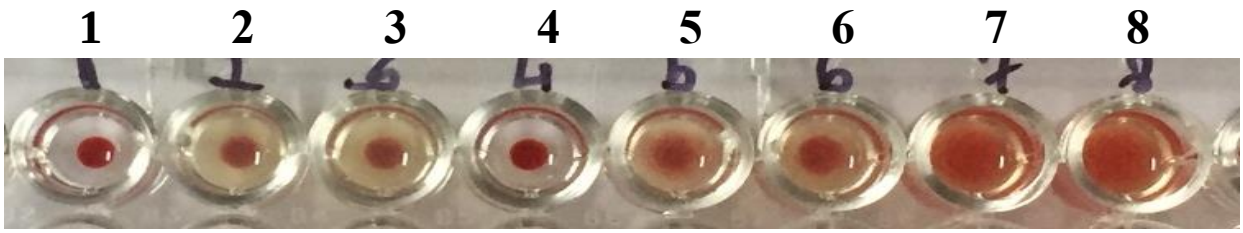
The FACS result of the Sample 3 and 4 including RBC + Anti CD59 + Anti CD59 + NHS + Anti C3b in without EGTA buffer.

The figure A depicts the black spot and red dots obtained representing the presence of granulocyte which are RBCs. Red spots represent the C3b deposition. Figure B depicts the gates that are created which shows 58.9.% C3b deposition. In figure C a larger shift in the histogram peak is observed.

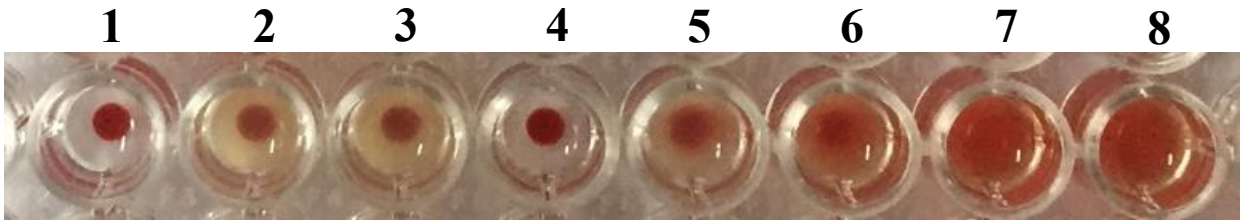
From the above figures it is concluded that more C3b deposition is observed in the case of without EGTA buffer as both the classical and alternative complement pathways are activated whereas in the case of with EGTA comparatively less deposition is seen as only classical pathway is working.

5.3 Lysis Assay

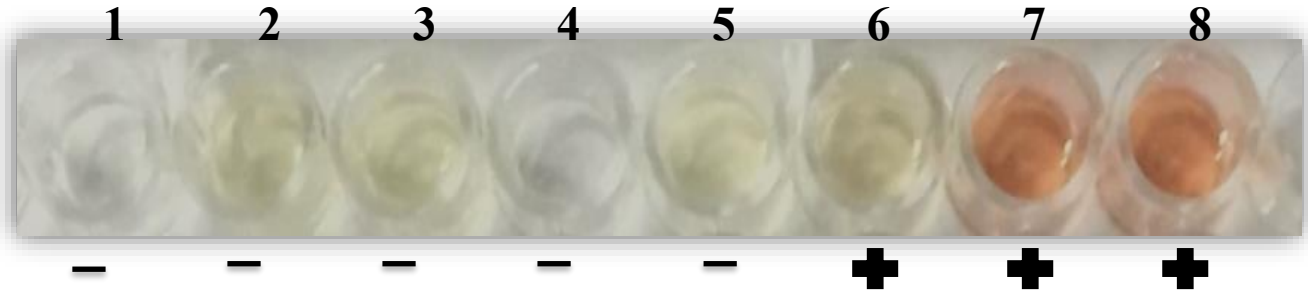
Lysis of RBCs is estimated in the assay. Due to the blocking of the receptors CD55 and CD59 (protectin) the RBCs undergo lysis by the classical and alternative pathway. The button formation is observed at the bottom of each well. Later carefully the supernatant is taken in another ELISA plate and the reading is taken at 405nm in the ELISA plate reader. The wells are labelled according to the details given in Table 7. A clear button formation is seen in well 1,2,3 and 4. In case of well 5 and 6 both button formation and lysis are observed. These wells consist of the antibodies Anti CD55 and Anti CD59 respectively. Antibodies are specific but it might be so that it doesn't bind to all the RBCs present in the well and some of which are left which undergo button formation. Some RBCs which are bound to the antibodies show lysis. Thus, well 5 and 6 depict both button formation and lysis. The last two wells well 7 and 8 clearly shows the lysis of the RBCs.



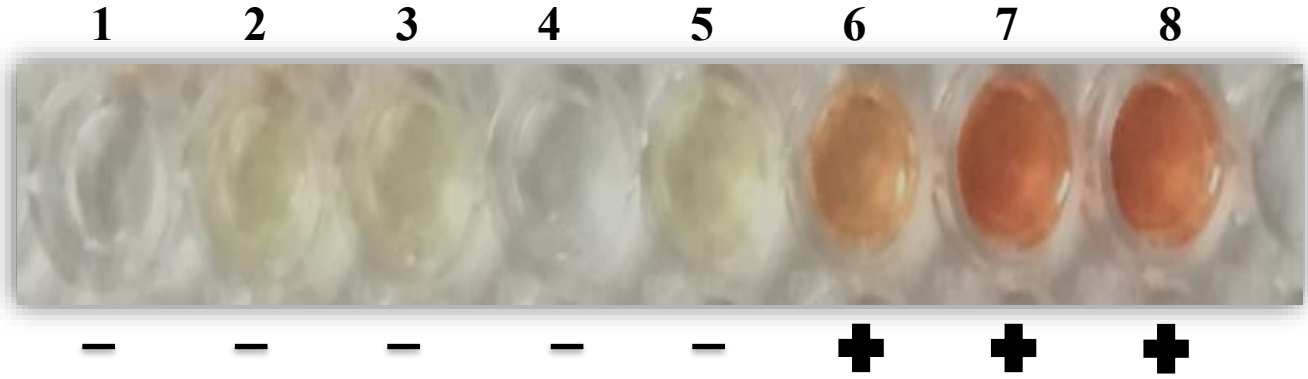
a) WITH EGTA



b) WITHOUT EGTA



a) With EGTA



b) Without EGTA

Figure 19: Lysis Assay and depiction of button formation at the base of the well is observed due to accumulation of the whole RBCs.

Table 7 : Composition of wells in the lysis plate

Sr. No.	WITH EGTA	O.D. at 405 nm	WITHOUT EGTA	O.D. at 405 nm
1.	RBC	0.071	RBC	0.067
2.	RBC + NHS	0.285	RBC + NHS	0.288
3.	RBC + NHS + Anti C3b	0.284	RBC + NHS + Anti C3b	0.274
4.	RBC + Anti C3b	0.059	RBC + Anti C3b	0.055
5.	RBC + Anti CD 55 +NHS + Anti C3b	0.283	RBC + Anti CD 55 +NHS + Anti C3b	0.293
6.	RBC + Anti CD 59 +NHS + Anti C3b	0.500	RBC + Anti CD 59 +NHS + Anti C3b	2.006
7.	RBC + Anti CD 55 + Anti CD 59 + NHS + Anti C3b	2.668	RBC + Anti CD 55 + Anti CD 59 + NHS + Anti C3b	3.123
8.	RBC + Anti CD 55 + Anti CD 59 + NHS + Anti C3b	2.723	RBC + Anti CD 55 + Anti CD 59 + NHS + Anti C3b	3.145

O.D obtained at 405nm are increasing in an ascending manner from sample 1 to sample 8. Thus, it can be said that as we go from sample 1 to 8 lysis of RBCs is observed. O.D is in range (0-1) in case of sample 1 to 6 as they are the controls. In case of sample 7 and 8 large values of O.D are obtained due to presence of color and thus, signifying the lysis of the cells.

Conclusions

6. CONCLUSION

- RBC lysis was analyzed visually and as well as using the Florescence Activated Cell Sorting (FACS).
- The receptor CD 55 binds to the C3 convertase inhibiting the complement pathway and receptor CD 59 inhibits the MAC formation.
- When Anti CD 55 is administered the lysis of the cells is very low.
- However, when Anti CD 55 and Anti CD 59 are administered together, high amount of cell lysis and C3b deposition is observed in the case of experiment without EGTA buffer (58.9%) whereas in the case of experiment with EGTA buffer it was comparatively lower (12.4%).
- EGTA activates only the classical complement pathway, while in experiments without EGTA, both classical and alternative complement pathway is affected. Thus, providing more C3b deposition and cell lysis in case of without EGTA buffer.

References

8. REFERENCES

1. Owen J.A., Punt J. and Stanford S.A. (2013). Kuby Immunology (seventh edition). The Complement System. United States of America: (W.H. Freeman).
2. http://www.complentsystem.se/Complement%20System
3. Moiola, M., Memeo, M. G., & Quadrelli, P. (2019). Stapled peptides-a useful improvement for peptide-based drugs. *Molecules*, *24*(20). https://doi.org/10.3390/molecules24203654.
4. Risitano, A. M. (2013). Paroxysmal nocturnal hemoglobinuria and the complement system: Recent insights and novel anticomplement strategies. *Advances in Experimental Medicine and Biology*, *734*, 155–172. https://doi.org/10.1007/978-1-4614-4118-2_10.
5. https://www.hopkinsmedicine.org/kimmel_cancer_center/types_cancer/paroxysmal_nocturnal_hemoglobinuria_PNH.html
6. Parker, C. J., Baker, P. J., & Rosse, W. F. (1982). Increased enzymatic activity of the alternative pathway convertase when bound to the erythrocytes of paroxysmal nocturnal hemoglobinuria. *Journal of Clinical Investigation*, *69*(2), 337–346. https://doi.org/10.1172/JCI110457.
7. https://www.aatbio.com/resources/assaywise/2017-6-2/a-practical-guide-for-use-of-pe-and-apc-in-flow-cytometry
8. Archives, Y., & Posts, B. T. O. (2018). *Recent blogs Let ' s Talk*. *3*, 2019.
9. Brodsky, R. A. (2019). Paroxysmal nocturnal hemoglobinuria without GPI-anchor deficiency. *Journal of Clinical Investigation*, *129*(12), 5074-5076.