INVESTIGATIONS IN THE AETIOLOGY AND PATHOPHYSIOLOGY OF SAGO HAEMOLYTIC DISEASE

Thesis submitted by
Numaya RAI (Gurung) BSc (MLS), TU, IOM (NEPAL)
in
December, 2004

in partial fulfilment of the requirements
for the degree of Master of Biomedical Sciences (Medical Lab Science)
at the School of Veterinary & Biomedical Sciences
James Cook University, Townsville, Australia

STATEMENT OF SOURCES

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

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ACKNOWLEDGMENTS

I sincerely thank my supervisor, Dr Jeffrey Warner for his encouragement and invaluable guidance during both the experimental and writing stages of this thesis.

I am grateful to A/Professor Warren Shipton for allowing me to work in his project and financial support.

Special thanks to Dr Wayne Melrose for his kind support and expert opinion in haematological aspect.

My personal thanks are expressed to Andrew Greenhill for his continuous support and advise during the experimental works and for providing me beautiful pictures of sago processing in PNG.

I wish to thank Laurie Reilly for providing me haematological staining reagents and required stuffs. Also Jenny Elliman for her kind cooperation.

I would like to thank the staff and students of the School of Biomedical science, James Cook University for their kind cooperation.

Special thanks to Linda Warner for her kind contribution in this project.

I am grateful to the member of "PNG WANTOK CLUB", student association of PNG at James Cook University, and their families and relatives for their generous coordination and participation. Without their cooperation, completion of this work was virtually possible. I would like to acknowledge the support and friendship from Eare Forova, Daisy Raburabu and Taita Viri.

Finally, I wish to thank my husband Ganesh and two sons, Gahan and Mohit for their moral support and encouragement.

ABSTRACT

Sago haemolytic disease (SHD) is a suspected mycotoxocosis that causes acute and sometime fatal intravascular haemolysis and has only been described in Papua New Guinea (Taufa, 1974; Donovan *et al.*, 1977). Although much is yet to be revealed about this condition, the main risk factor is the consumption of stale sago starch, a food stuff which is the staple carbohydrate for rural Papua New Guineans. It is thought that fungi that colonise the sago are responsible for the haemolytic compound that cleaves red cell membrane proteins. The condition often manifests in family clusters and a genetic predisposition has been postulated. Melanesian Ovalocytosis (MO), also known as South East Asian Ovalocytosis (SAO), is a autosomal recessive genetic disorder expressing a red cell membrane band 3 deletion in individuals who have inherited the gene responsible. The condition is subclinical but common in rural PNG where it is thought to offer some malaria protection (Bruce *et al.*, 2000). It is reasonable to assume that as this condition is widespread in rural PNG where SHD is endemic, it may a confounding predisposing factor to the manifestation of the acute haemoltyic crisis.

A number of organisms implicated in SHD have been isolated and crude haemolytic compounds were isolated as per methods of Bernheimer (Bernheimer, 1988). Red cell membrane ghosts where prepared from individuals with and without the MO band 3 deletion and exposed to the toxins. Proteins were separated using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the activity of the toxin was demonstrated by the cleaving of band known to be present through comparison with untreated controls. Although cleavage of bands was demonstrated by various toxin compounds no discernable difference could be identified between the two groups. This may suggest that MO is not involved as a host factor which predisposes SHD, although further studies are required to elucidate this finding.

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ABBREVIATIONS

B. cereus	*Bacillus cereus*
BA	blood agar
C. perfringens	*Clostridium perfringens*
C.albicans	*Candida albicans*
CPA	carrot potato agar
DDT	diothiothreitol
DLF	direct lytic factor
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
gm	gram
g	gravity
G6PD	glucose 6 phosphate dehydrogenase
GSH	glutathione
H. influenzae	*Haemophilus influenzae*
H_2O_2	hydrogen peroxide
Hb	haemoglobin
HIV	human immuno deficiency virus
HMP	hexose mono phosphate shunt
hr	hour
HUS	haemolytic uremic syndrome
IV	intravenous
K^+	potassium
kDa	kilo dalton
l	litre
L. monocytogenes	*Listeria monocytogenes*
M	molar
min	minute
ml	milli litre
mM	milli molar
MO	Melanesian ovalocytosis
µl	micro litre
µm	micro metre
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nano metre
^oC	degree Celcius
OMPLA	outer membrane phospholipase A
P. steckii	*Penicillium steckii*
P. citrinin	*Penicillium citrinin*
P. sizovae	*Penicillium sizovae*
P.aeruginosa	*Pseudomonas aeruginosa*
PAT	platelets activating factor
PBS	phosphate buffered saline
PC	phosphotidylcholine
PCR	polymerase chain reaction

PE	phosphatidylethanolamine
PG	phosphotidylglycerine
PI	phosphotidylinositol
PNG	Papua New Guinea
PS	phosphatidylserine
RBC	red blood cell
RBCM	red blood cell membrane
rpm	revolution per minute
S. aureus	*Staphylococcus aureus*
S. typhimurium	*Salmonella typhimurium*
S. pneumoniae	*Streptococcus pneumoniae*
SC	subcutaneous
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp	species
TDS	toxin diluting solution
Y. enterocolitica	*Yersenia enterocolitica*

CHAPTER 1 - INTRODUCTION

Sago haemolytic disease is fatal and common in Papua New Guinea (PNG). Several outbreaks of this intravascular haemolytic anaemia have been reported (Taufa, 1974; Donovan *et al.*, 1977). Previous workers have attempted to establish the relationship between consumption of stale sago and this disease. It is found that in those outbreaks, patients were either from the same family or they had shared the same batch of sago.

From this finding, it is indicative that some types of genetic disorders may have an important role and act as a predisposing factor for the occurrence of this disease.

It is suggested that due to the high frequency of G6PD deficiency in those endemic areas, it could be the predisposing factor for the disease, although earlier observations suggest that the haemolytic effect is not oxidative in nature and therefore unlikely to be linked with G6PD deficiency (Donovan *et al.*, 1977). Melanesian ovalocytosis (MO) is a common hereditary disorder (up to 40%) of individuals within these regions (Bruce *et al.*, 2000). Hence, it led us to hypothesize whether MO could be predisposing factor for SHD in people of PNG.

Several organisms have been isolated from sago and they are known to possess toxins containing haemolytic factors and some of them are found to be capable of damaging red blood cell membrane (RBCM). Melanesian ovalocytosis is itself a disorder of RBCM, so possibly these haemolytic toxins from sago may damage the RBCM of MO more severely as compared to normal RBCM.

This work includes various procedures and methods. After human ethics approval, the participants were recruited and written consent was obtained from them. Students at James Cook University and their family members from PNG (30), Indonesia (4), and Malaysia (1) were selected randomly as participants.

Screening was done by taking blood by finger prick, preparing smear and examining after staining. Venous blood was obtained from those participants who were

identified as ovalocytosis and the blood sample was immediately processed for ghost cell preparation.

Toxins were extracted from those organisms, which were isolated and identified from the sago sample collected from PNG and their haemolytic activity were determined by haemolytic assay.

Finally, the ghost cells prepared from blood drawn from the positive cases, were treated with toxins that was extracted from the different organisms and their effects were demonstrated by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

CHAPTER 2 - LITERATURE REVIEW

2.1 Introduction

The red blood cell (RBC) is well known for its ability to withstand great deformations when it is passes through micro capillaries (Lenormand *et al.*, 2001). A normal human RBC assumes a biconcave discoid shape ~ 8 μm in diameter (Lim *et al.*, 2002). The normal life span of RBC is 100 to 120 days (McKenzie, 1996). However, there is a wide range of influencing factors that cause premature destruction of RBC. This premature destructing phenomenon is known as haemolysis (McKenzie, 1996). Haemolysis can be categorized in different groups depending on either its occurring sites or aetiology. Haemolysis can be either intra vascular (within the circulation) or extra vascular (within the tissue macrophages) (Rodak, 2002). Laboratory investigation is very useful and very important tool for the detection and determination of haemolysis. Extra vascular haemolysis is more common than intra vascular haemolysis (McKenzie, 1996). Ninety percent of normal RBC degradation occurs extra vascularly (Rodak, 2002).

A large body of work has postulated that there are numerous aetiologies of haemolysis. However the aim of this literature review is to describe and discuss the various microbial causes and possible mechanism of the red blood cell destruction.

2.2 Erythrocyte Membrane

2.2.1 Membrane composition

The erythrocyte membrane is composed of 52% protein, 40 % lipid, and 8 % carbohydrate. This biphospholipid protein complex regulates the membrane functions of transport and flexibility and determines the antigenic properties of membrane.

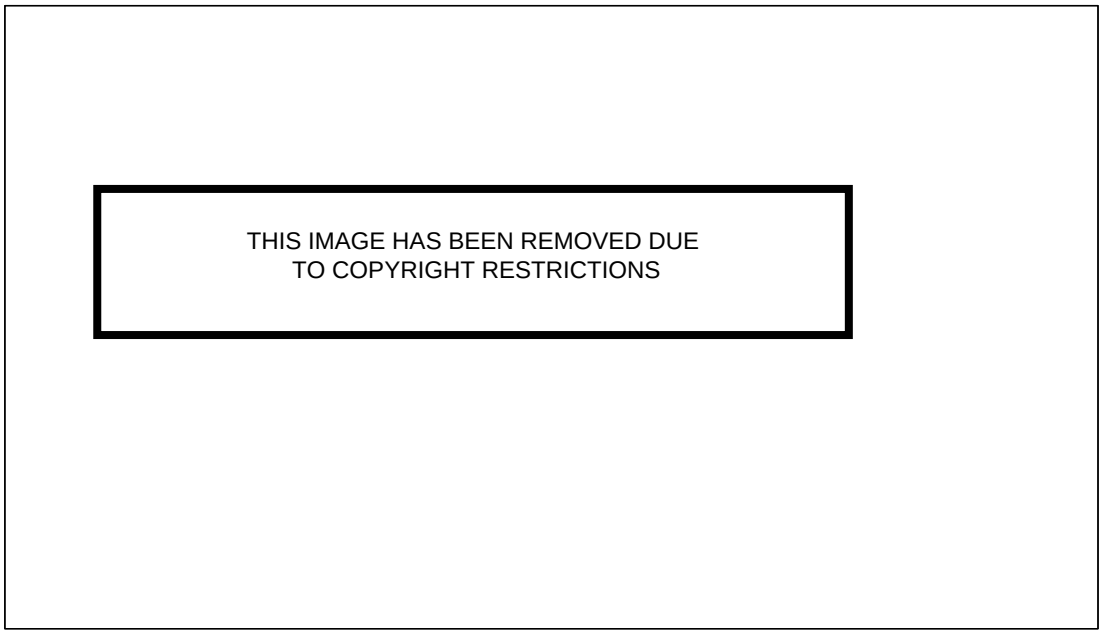


Figure 2.01 Schematic representation of red cell membrane architecture reproduced from Picart and Discher (1999).

Table 2.01 Erythrocyte Membrane Composition

Lipids	Protein
• Unesterified cholesterol • Phospholipids ▹ cephalin ▹ lecithin ▹ sphingomyelin ▹ phosphatidylserine • Glycolipids	• Integral proteins ▹ glycophorins A,B,C ▹ band 3 • Peripheral proteins ▹ spectrin ▹ actin ▹ ankyrins ▹ band 4.1 ▹ band 4.2 ▹ band 4.9(dematin) ▹ trypomysin

The red blood cell membrane (RBCM) is one of the most thoroughly researched structures in biology (Heinrich *et al.*, 2001). The RBCM is a primary model for animal cell plasma membranes (Cho *et al.*, 1999; Zhang *et al.*, 2000). The RCBM is

composed of a lipid bilayer reinforced on its inner face by a flexible two dimensional protein network (Yawata *et al.*, 1997; Cho *et al.*, 1999; Lenormand *et al.*, 2001). The outer lipid bilayer consists of almost equal amount of phospholipids and non-esterified cholesterol and a small amount of glycolipids (McKenzie, 1996).The majority of the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are located in the inner leaflet whereas the cholinphospholipids, phosphatidlycholine and sphingomyelin are localized predominantly in the outer leaflet (Manno *et al.*, 2002).

The phospholipid molecules are arranged with polar heads facing to the inside and outside of the cell and the hydrophilic tails facing to the interior of bilayer (McKenzie, 1996). The cholesterol is embedded in this leaflet lipid bilayer and stabilises it. Glycolipids are also embedded here and located entirely in the external half of the bilayer. Several important RBC antigens, including A, B, H, and P are located in the lipid bilayer (Rodak, 2002). The inner cell is only composed of a viscous fluid (solution of haemoglobin), the resistance to the stress is mainly attributed to the elastic properties of its membrane (Heinrich *et al.*, 2001; Lenormand *et al.*, 2001).

The integral proteins penetrate across the lipid bilayer and can interact with hydrophobic lipid area (Rodak, 2002). These proteins are usually identified by a number according to their separation by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Integral proteins consist of two types: glycoprotein and band 3 protein (McKenzie, 1996). Peripheral membrane proteins interact with protein or lipids at the membrane surface but do not penetrate the bilayer area. They line the inner membrane surface and interact to form a “membrane skeleton” or “cytoskeleton” (Rodak, 2002).

This skeleton is made of spectrin dimers associated to form mainly tetramers ~ 200 nm long. They are linked together by complex junctions (primarily composed of actin filaments and protein 4.1) and attached to lipid bilayer by transmembrane protein (glycoprotein and band 3) (Lenormand *et al.*, 2001).

The protein portion of the membrane is responsible for the shape, structure, and deformability of the RBC. Direct protein- lipid associations have been also suggested to be involved in the coupling of this skeleton to the membrane. In certain haemolytic diseases loss of membrane lipids and production of non deformable spherocytic cells may result from uncoupling of the skeleton from the membrane (Shahrokh *et al.*, 1991).

The interaction of the cytoplasmic domain of a sub population of band 3 oligomers with the membrane skeleton is critical for maintaining normal erythrocyte shape and mechanical properties (Blackman *et al.*, 2001).

2.2.2 Membrane function

It is absolutely essential to have a normal intact membrane for normal erythrocyte function and survival. Any kind of abnormalities either inherited or acquired in membrane structure or composition may result in severe anaemia. During the beginning of the 20th century, studies began which established the complexity of the erythrocyte membrane. The experiments performed by Hedan demonstrated the osmotic properties and selective permeability of the erythrocyte. His experiments demonstrated that erythrocyte volume increased in hypotonic solution of sodium chloride or sucrose, however, caused shrinkage of the cell (McKenzie, 1996).

Several years later, Landsteiner discovered the antigenic properties of the membrane. He found that human sera caused agglutination of the erythrocytes in different individuals. Originally he categorised these individuals into three groups A, B, and C, according to their erythrocyte agglutination patterns with human sera. Now, the terminology, group A, B, and O is widely used to identify blood groups on the basis of the agglutination patterns. Apart from these antigens, there are hundreds of erythrocyte antigens which were discovered since 1940 (McKenzie, 1996).

Research of blood circulation has revealed that the 7μm erythrocyte must be deformable (flexible) to squeeze through the micro 3μm diameter channel of the capillaries of the spleen. The deformability of the erythrocyte membrane and fluidity

(mainly haemoglobin) of the cells content are the important properties of the erythrocyte membrane. Deformability is a reversible mechanism of membrane, which occurs when the cell changes geometric shape without the change in surface area. Any kind of decreases in deformability of membrane or fluidity of content leads to decreased erythrocytic deformability. Then the abnormal cell will be trapped in the splenic cords and is destroyed by macrophages.

These cells with decreased deformability are also prone to fragmentation even under the normal stress of circulation. In other words, the erythrocyte could be compared with a plastic sandwich bag only partially filled with water. Because of the deformability of the plastic and the fluidity of the contents, the shape of the bag could be altered in various forms. On the contrary, if the bag is made of glass and if the content is frozen, obviously, the bag will lose deformability and break under the force which tend to distort it (McKenzie, 1996).

Therefore, it is clear now that the main physiologic functions of the RBC membrane are:
(1) to maintain cell shape deformability for osmotic balance between plasma and the cell cytoplasm, (2) to act as a supporting skeletal system for surface antigens, and (3) to help in the transportation of essential ions and gases. Transportation is divided in two types, (1) Passive transportation, which occurs by simple diffusion through cell pores (gases, glucose), and (2) active transportation which involves the movement of substances and electrochemical gradient (Na^+, K^+) (Rodak, 2002).

2.2.3 Red blood cell metabolism

2.2.3.1 Hexose- monophosphate shunt

When glucose enters the cell, it is catabolized via the Embeden- Meyerhof (EM) pathway or the hexose- mono- phosphate shunt (HMP). Hexose- monophosphate shunt catabolizes ~ 10 % of the glucose, which is essential for maintaining sufficient concentration of reduced glutathione (GSH) through the production of NADPH. Glutathione is the important cellular antioxidant, which maintains haemoglobin in

the reduced functional state and preserves vital cellular enzymes from oxidant damage. When the cell is exposed to an oxidizing agent, the production of NADPH increases.

If enzymes in this pathway are incompetent, oxidised haemoglobin accumulates and denatures to form Heinz bodies. Heinz bodies attach to the RBC membrane, causing increased membrane permeability to cations, osmotic fragility, and cell rigidity. These abnormal cells become trapped and haemolysed in the spleen. G6PD deficiency is an X - linked hereditary disorder, and the most common of all clinically significant enzyme defects (Oliver *et al.*, 2001), which can lead to acute haemolytic anaemia following ingestion of fava beans and certain drugs, and bacteria or virus (Meloni *et al.*, 1983). The role of infections as a cause of haemolysis by oxidation stress has been most thoroughly studied in individual with G6PD deficiency.

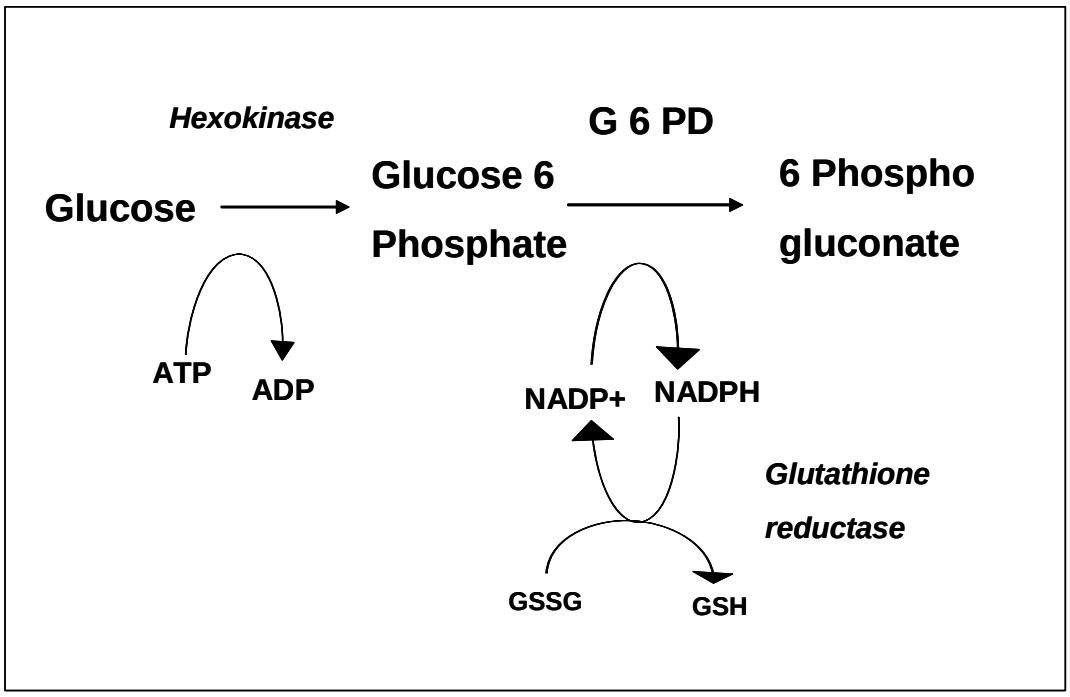


Figure 2.02 The Hexose Monophosphate Shunt

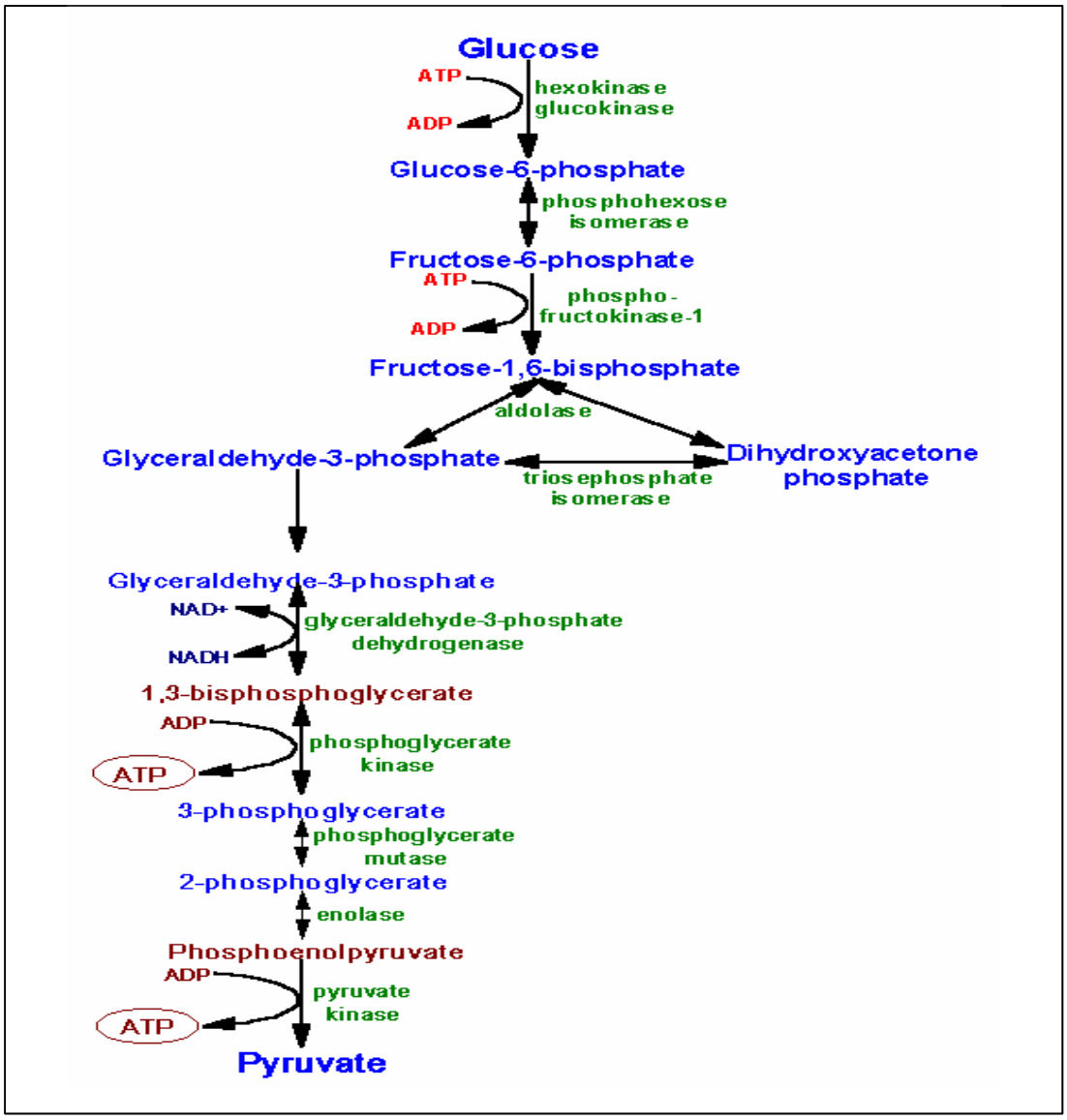


Figure 2.03 Embden – Meyerhof PathwayReproduced from Michael W. King, PhD / IU School of Medicine mking@medicine.indstate.edu

2.2.4 Regulation of red blood cell membrane integrity

In spite of the numerous hard works and recent progress in understanding the structure and function of biological membranes, some important issues are still unsolved. It is still unclear how the specific lipid composition of the cellular membranes arise and are maintained (Virtanen *et al.*, 1998).

The shape and deformability of human RBCM is thought to be maintained by a complex of protein- protein and protein- lipid interactions (Picart *et al.*, 2000; Khanna *et al.*, 2002). Among them, the well- defined complexes are associations

within the spectrin-actin skeleton, usually termed as horizontal interactions, and bridge between the membrane skeleton and various membranes spanning proteins known as vertical interactions.

The horizontal interactions are considered important, because they maintain the architecture and stability of the protein network underlying the lipid bilayer. The vertical interactions are thought to be essential, since they permit stress sharing between the lipid bilayer and protein skeleton, and they prevent aggregation of membrane – spanning proteins and the consequent vesiculation of protein free lipid domains (Chang and Low, 2001; Manno *et al.*, 2002).

Even though the functions of spectrin, actin, and protein 4.1 have been extensively characterized, physiological function is still undefined (Chang and Low, 2001; Khanna *et al.*, 2002; Manno *et al.*, 2002). Further investigation of the interaction between the bilayer part of the membrane and cytoskeleton/ membrane skeleton is required to understand the long range structural basis of the functions of the plasma membrane (Takeuchi *et al.*, 1998).

A condition known as haemolytic anaemia develops due to the excess RBC destruction. Haemolytic anaemia may be classified as either as inherited or acquired. Moreover, it can be further classified as haemolytic anaemia with intrinsic defects or extrinsic defects according to the aetiology of haemolysis. The majority of intrinsic defects are hereditary and an abnormality of the RBC itself. It can be structural defect in RBC membrane, haemoglobin molecule or in enzymatic pathways. On the other hand, extrinsic defects are mostly acquired. The RBC is damaged by various types of chemical; mechanical and physical action and infectious agents. Among the external factors associated with haemolytic activity are snake venom (Quiros *et al.*, 1992), and insects (Deregnaucourt and Schrevel, 2000).

2.3 Clinical Signs and Symptoms of Haemolytic Anaemia

Haemolytic anaemia is associated with increases in both haem catabolism (RBC destruction) and erythropoiesis. Jaundice is a prominent reflection of an increase in bilirubin production. Gallstones consisting primarily of bilirubin are common in congenital haemolytic anaemia. Dark or red urine due to excretion of plasma haemoglobin may be noted in intravascular haemolysis (McKenzie, 1996).

The primary symptoms associated with anaemia include pallor, fatigue, and cardiac symptoms. Chronic severe haemolytic anaemia stimulates the expansion of bone marrow, consequently thinning corticol bone and widening the spaces between inner and outer tables of bone. In children, the expansion indicates skeletal abnormalities. These bone changes may result in spontaneous fractures and a type of arthritis termed “osteoarthropathy”. Splenic hypertrophy is a constant finding of extravascular haemolysis.

Table 2.03 Classification of haemolysis

Anaemia characterised by intra vascular haemolysis	**Anaemia characterised by extra vascular haemolysis**
1. Activation of complement on the RBCM ▸ Paroxysmal nocturnal haemoglobinuria ▸ Paroxysmal cold haemoglobinuria ▸ Some transfusion reactions ▸ Some auto immune haemolytic anaemia 2. Physical or mechanical trauma to RBC ▸ Microangiopathic haemolytic anaemia ▸ Abnormality in heart and great vessels ▸ Disseminated intravascular coagulation 3. Toxic microenvironment ▸ Bacterial infection ▸ *P. falciparum* infection ▸ Venoms ▸ Arsenic Poisoning ▸ Intravenous administration of dH20	1. Inherited RBC defects ▸ Thalassemia ▸ Haemoglobinopathies ▸ Enzyme deficiencies ▸ Membrane defects 2. Acquired RBC defects ▸ Megaloblastic anaemia ▸ Spur cell anaemia ▸ Vit.E deficiency in new born 3. Immunohaemolytic anaemia ▸ Autoimmune ▸ Drug induced

Table 2.04 Classification of haemolytic anaemia

Intrinsic (Inherited)	**Extrinsic (Acquired)**
Membrane defects	Antagonistic plasma factors
Hereditary spherocytosis	Chemicals, drugs
Hereditary elliptocytosis	Animal venoms
Hereditary pyropoikilocytosis	Infectious agents
Hereditary stomatocytosis	Plasma lipid abnormalities
Hereditary xerocytosis	
PNH (Acquired)	Traumatic physical cell injury
	Microcirculation lesions
Enzyme disorders	Thermal injury
G6PD deficiency	March haemoglobinuria
Pyruvate kinase deficiency	
	Immune mediated cell destruction
Abnormal haemoglobins	Autoimmune
Thalassemia	Alloimmune
Structural haemoglobin variants	Drug- induced

Anaemia is the most prominent feature during the course of infection, both in acute and chronic infections in children. Failure of iron utilization, bone marrow suppression, and shortened duration of red blood cell survival are possible mechanisms of anaemia development.

Haemolysis is important feature of infections such as malaria but there are a large number of microorganisms, which can cause haemolysis. Therefore, it is important to recognise the haemolysis, which could be great help for the specific management and identification of cause of the infection process. Clinically useful classification of different aetiology and association of haemolysis and infections process has been reviewed (Berkowitz, 1991).

Table 2.05 Categories of association of infection and haemolysis (Berkowitz,

1991).

I. Infection causing haemolysis - no underlying RBC abnormality i. Infection of the RBC: malaria; babesiosis; bartonellosis ii. Haemolytic toxins: haemolysins; phospholipases iii. Immune haemolysis ▸ Auto antibodies to the RBC ▸ Deposition of immune complexes on the RBC ▸ Exposure of T antigens in the RBC membrane iv. Congenital infections: unknown mechanism
II. Infection precipitating haemolysis by oxidative stress in individuals with underlying i. RBC disorder: G6PD deficiency ii. Unstable haemoglobin iii. Paroxysmal nocturnal haemoglobinuria
III. Infection-induced pathologic responses causing haemolysis i. Haemolytic uremic syndrome (HUS) ii. Cardiac haemolytic anaemia iii. Hypersplenism
IV. Haemolysis caused by anti infective therapy i. Auto immune ii. Oxidant stress iii. Toxic, e.g., ribavirin

V. Underlying haemolytic disorder predisposing to infection i. Hyposplenism: sickle cell disease ii. Ischaemia: sickle cell disease iii. Reticuloendothelial blockade iv. Hyperferremia v. Defect in neutrophil killing variant of G6PD deficiency
VI. Predisposition to infection by therapy for haemolytic disorders i. Blood transfusion: transmission of infectious agents ii. Immunosuppressive therapy iii. Splenectomy iv. Iron overload from repeated blood transfusion v. Deferoxamine therapy: predisposition to systemic yersiniosis, zygomycoses
VII. Underlying haemolytic disorder, with aplastic crisis precipitated by infection with parvovirus B19.

2.4 Infections Causing Haemolysis

2.4.1 Malaria

Severe anaemia is one of the most lethal complications in children infected with *Plasmodium falciparum* (Waitumbi *et al.*, 2000). *Plasmodium falciparum* is responsible for the death of more than one million people annually (Woodrow *et al.*, 2000; Garratty *et al.*, 2002; Weatherall *et al.*, 2002). Haemolysis and a fever should always be considered in patients for the diagnosis of malaria. Haemolysis and defective erythropoiesis are cause of anaemia in malaria. Haemolysis occurs mainly extravascular but may be intravascular in severe cases (Berkowitz, 1991). The mechanisms of haemolysis appear to be both structural and immune. The structural alterations in the RBC include membrane damage and increased membrane rigidity (Berkowitz, 1991).

The pathogenesis of severe anaemia in malaria is unclear but it may result from multiple poorly understood processes including acute haemolysis of uninfected RBCs and dyserythropoiesis, as well as through the interaction of malaria infection with other parasite infections, and nutritional deficiencies (Weatherall *et al.*, 2002). The presence of circulating monocytes containing phagocytosed infected RBCs indicate that during malarial infection, uninfected cells develop lesions that activate monocytes to capture and engulf them.

Another possible mechanism of RBC destruction is through the activation of complement, which may occur in malarial infection (Waitumbi *et al.*, 2000). Anaemia may result from an immune- mediated process, antimalarial antibodies and compliment reacting with malarial antigens on erythrocyte membrane. This will results in removal of the sensitized cells by splenic macrophages (McKenzie, 1996).

In malaria infection, the presentation of severe intravascular haemolysis associated with haemoglobinemia, methaemalbuminemia, hyperbilirubinemia, haemoglobinuria are characteristics of disorder known as “Black water fever”. Finally it will result in renal failure. Black water fever has an association with the irregular use of quinine (McKenzie, 1996; Weatherall *et al.*, 2002). Anti-malarial drug quinine may act as hapten and stimulate production of a drug dependent complement- fixing antibody (Weatherall *et al.*, 2002).

Previously, Europeans who have taken quinine irregularly have been susceptible (McKenzie, 1996). However, recently, studies have shown that “black water fever” appears also common in Southeast Asia and PNG but rare in Africa. The use of different anti-malarial drugs such as, quinine, mefloquine, and artesunate, and G6PD deficiency are suggested to be the cause of the complication (Weatherall *et al.*, 2002). Malaria parasites oxidise RBC NADPH from the pentose phosphate pathway for its metabolism leading to the peroxide – induced haemolysis.

The malaria infection can be confirmed by detecting parasites in Giemsa – stained thick or thin smears. However, various advanced methods can be implemented for the diagnosis of the infections. Polymerase chain reaction (PCR), fluorescent stains

(QBC), and automated analysers are some examples of modern methods that are more confirmative but not affordable to small laboratory (Weatherall *et al.*, 2002).

2.4.2 Babesiosis

Babesiosis is an infection of animals that may be occasionally transmitted to human by *Ixodes scapularis*, a deer- tick (Berkowitz, 1991; Rodak, 2002; Herwaldt *et al.*, 2003). *Babesiosis microti* is human pathogen which may be confused with malaria parasite on microscopic examination (Berkowitz, 1991; Rodak, 2002). The disease is self- limiting but it may be fatal in splenectomised individuals. The parasites can be observed as an intracellular pleomorphic ring shaped inclusions on Romanowsky – stained peripheral blood smears (McKenzie, 1996; Rodak, 2002). The mechanism of individual haemolysis in babesiosis is not clear. Proteins, inclusions, and perforations are the changes in RBC membrane induced by parasite. Haemophagocytosis in the bone marrow has also been mentioned (Berkowitz, 1991).

2.4.3 Bartonellosis

Bartonellosis is caused by gram negative bacilli *Bartonella bacilliformis* and is transmitted by sand fly of the genus *Lutzomyia* (Berkowitz, 1991; Ellis *et al.*, 1999; Rodak, 2002; Maco *et al.*, 2004). It comprises of two phases, an acute bacteremia with severe febrile haemolytic anaemia (Oroya fever) and the chronic phase with nodular skin lesions, Peruvian wart (verruga peruana) (Ellis *et al.*, 1999; Maco *et al.*, 2004). This disease is mainly found in Colombia, Peru, and Ecuador. The parasites can be demonstrated either as intracellular or extracellular on Romanowsky stained peripheral blood smears (McKenzie, 1996). The organism infects endothelial cells and attached to the surface of RBCs. The acute phase of the infection is associated with a severe haemolytic anaemia, and the chronic phase is characterised by verrucosa skin lesions. It is been suggested that the causative organism can be removed from the RBC surface by phagocytes without damaging the RBC (Berkowitz, 1991).

2.5 Haemolytic Toxins

Many pathogenic microorganisms produce haemolytic toxins (haemolysins). Haemolysins are closely associated with the virulence of a number of bacterial pathogens. The modes of action of haemolysins are variable, but there are several studies which have been performed which define the ability of bacteria to lyse RBCs with phospholipase activity (Grant *et al.*, 1997).

A large number of haemolytic toxins (haemolysins) are produced by many pathogens and are also present in living cells, not all toxins are responsible for producing *in vivo* haemolysis in human and cause systemic haemolysis. For instance, haemolysins produced by streptococci, staphylococci, and many other bacteria can produce haemolysis surrounding the colonies on blood agar plates when cultured in vitro. Therefore, it is understood that these haemolysins have contribution in virulence of pathogens rather than in anaemia. On the other hand, phospholipases A_2 enzymes present in venoms and toxins have potency to rupture the erythrocytes in our system and cause haemolytic anaemia.

Staphylococcus aureus produces four haemolytic toxins, α, β, γ, and δ haemolysins (Jeljaszewicz *et al.*, 1978a). *Staphylococcus aureus* causes a broad range of life threatening diseases in humans. Alpha - toxin is thought to be a major virulence factor (Worlitzsch *et al.*, 2001). Alpha- toxin has haemolytic, dermo- necrotic, and lethal properties. This is the most studied of the staphylococcal membrane damaging toxins.

However, its mechanism of action at the molecular level is still not completely understood. It has been suggested that alpha- toxin is surface active and its action involves penetration and disorganisation of the hydrophobic regions of the cell membrane. Haemolytic and cytolytic activities are closely associated with biological membrane damage. Alpha- toxin damages primary and established cell culture of various origins and causes their subsequent death (Jeljaszewicz *et al.*, 1978a).

Substances that induce haemolysis of blood cells are secreted by a number of gram-negative and gram- positive bacteria. The role of haemolysins in the virulence of gram- negative pathogens has not been fully defined, but these agents serve as models for genetic analysis of exotoxins. Currently the haemolysis of *E. coli* (Gray *et al.*, 1988; Neil and Holmes, 1988; Chart *et al.*, 1998; Figueiredo *et al.*, 2003) and *P. aeruginosa* (Neil and Holmes, 1988) has been studied in the most detail.

Synthesis of α- haemolysin by *E. coli* is complex process involving interactions of several proteins. At least three different genes, designated hly A, hly B, and hly C, are involved in controlling the production of haemolysins (Gray *et al.*, 1988; Neil and Holmes, 1988). Although the role of *E. coli* haemolysis in pathogenesis is incompletely defined, genetic evidence supports the role of haemolysin as a virulence factor. The haemolysin was thought to provide iron for the bacteria by lysing red blood cells of the host. No specific role of haemolysin in *E. coli* is physiology, other than as a potential virulence factor, has been identified (Neil and Holmes, 1988; Figueiredo *et al.*, 2003).

Pseudomonas aeruginosa produces two extracellular haemolysins; a heat stable glycolipid (Neil and Holmes, 1988; Figueiredo *et al.*, 2003) and heat labile phospholipase C. The amount of phosphate available to that cell regulates the synthesis of phospholipase C (Mollyby, 1978; Neil and Holmes, 1988). Mutants deficient in phospholipase C or defective in depression of phospholipase and other phosphate regulated proteins have been isolated. A mutant that produces phospholipase C and other phosphate- regulated proteins constitutively has also been isolated.

These results suggest that the phosphate- regulated proteins are constituents of a general phosphate retrieval system, and the haemolysins are believed to function cooperatively with other factors to aid in obtaining phosphate from phospholipids in the environment (Neil and Holmes, 1988).

Leptospira interrogans (Lee *et al.*, 2002), *Mycobacterium avium* (Maslow *et al.*, 1999), and fungus *Stachybotrys chartorum* (Vesper *et al.*, 1999) are other important

haemolysin producing organisms.

2.5.1 Streptolysin O

Streptolysin Group A and G streptococci and human strains of group C produce streptolysin O which is a thiol – activated, membrane damaging protein of M_r 69,000 (Bhakdi and Tranum-Jensen, 1985; Wannamaker and Schlievert, 1988; Yamamoto *et al.*, 2001). The oxygen-labile haemolysins of groups C and G streptococci appear to be identical to streptolysin of group A streptococci, both immunologically and with other properties which have been examined. Streptolysin O also closely resembles the haemolysins of a variety of other gram-positive bacteria, such as the *S. pneumoniae*, the tetanus bacillus, and other clostridia, and *Listeria monocytogenes* (Bhakdi and Tranum-Jensen, 1985; Wannamaker and Schlievert, 1988). These lysins are stimulated by sulf- hydryl compounds, and inhibited by cholesterol in low concentrations. Basically, streptolysin O is found in the extracellular fluid and an identical haemolysin has been found in the periplasm also. Haemolytic units of streptolysin O have been defined in various ways. The measurement of 50 % haemolysis by colorimetric methods has been shown to be more accurate than serial dilutions with visual determination of an end point (Wannamaker and Schlievert, 1988).

Streptolysin O acts on cell membrane (Bhakdi and Tranum-Jensen, 1985; Bhakdi *et al.*, 1985; Wannamaker and Schlievert, 1988; Yamamoto *et al.*, 2001). Although most studies have been performed on red blood cells, the effects on other cells are probably similar (Jeljaszewicz *et al.*, 1978b). Unlike most other bacterial lysis, the rate of lysis of erythrocytes is a non- linear function of streptolysin O concentration. An initial short lag phase is followed by a constant- rate phase during which the haemolytic rate is proportional to the square of the concentration of erythrocytes is increased. Multiple hits may be required for haemolysis to occur or the efficiency of haemolysis may be increased by the cooperation of two molecules of streptolysin O bound to adjacent receptors. Some authors have suggested that only a few (two or three) molecules may be required to lyse a single erythrocyte.

Zinc ions inhibit an early step leading to haemolysis, apparently by preventing the binding of streptolysin O to the erythrocyte membrane. A considerable body of evidence suggests that cholesterol is the binding site for streptolysin O in nucleated mammalian cell as well as erythrocytes. Streptolysin O is active only on cells whose membrane contains cholesterol (Bhakdi and Tranum-Jensen, 1985; Wannamaker and Schlievert, 1988).

The steps after binding are dependent on temperature, ionic strength, and pH and are inhibited by divalent cations. Two distinct sites have been proposed to be involved in the interaction of streptolysin O with red blood cells. This hypothesis suggests that the ‘f’ or fixation site is responsible for initial adsorption, and the ‘t’ or toxic site (l or lytic site) has an important role in the subsequent events which result in lysis (Wannamaker and Schlievert, 1988).

However, the mechanism of the final events producing the lysis of erythrocytes by streptolysin O is undefined. In previous studies, it has been suggested that no osmotic mechanism is involved in the lysis by streptolysin O. These studies showed that ions and Hb are released almost simultaneously, and the amount of Hb released is directly proportional to the concentration of lysed cells, suggesting a rapid. However, recently, a study has suggested that colloid osmotic lysis is responsible for the haemolysis of red blood cells by streptolysin O (Wannamaker and Schlievert, 1988).

2.5.2 Streptolysin S

Streptolysin S is a cytolytic toxin responsible for the haemolysis surrounding colonies of group A streptococci on blood agar plates (Jeljaszewicz *et al.*, 1978a; Wannamaker and Schlievert, 1988). In contrast to streptolysin O, streptolysin S is cell- bound and is induced and released by various unrelated groups of substances such as serum or serum constituents (albumin and α- lipoprotein), RNA (RNA core), detergents, and different of aniline dyes (eg. trypan blue). These substances may serve as carriers for and stabilizers of the small polypeptide moiety that is the haemolytically active component of the complex. This haemolytic component can be

transferred from one carrier to another haemolysin, which is thought to be the precursor of streptolysin S, and has been demonstrated in the streptococcal cell. Streptolysin S is determined by its haemolytic activity depending on units, by utilising various methods by different researchers. Streptolysin S is resistant to trypsin and pepsin but is inactivated by chymotrypsin and pepsin (Jeljaszewicz *et al.*, 1978a; Wannamaker and Schlievert, 1988).

The mode of action of streptolysin S has been obtained from studies of its interaction with erythrocytes. The first step is temperature dependent, whereas further steps are independent of temperature. Streptolysin S damages the osmotic barrier of the cell membrane. An ion flux occurs followed by swelling of the erythrocytes and membrane rupture with release of haemoglobin (Jeljaszewicz *et al.*, 1978a; Wannamaker and Schlievert, 1988). For the production of the lytic effects, streptolysin S may reacts with some components of the cell membrane, probably, the phospholipids, but streptolysin S does not possess phospholipase activity and no lesion of the erythrocyte membrane can be demonstrated by electron microscopy (Wannamaker and Schlievert, 1988) .

2.5.3 Phospholipase A

Phospholipases are one of the most important enzymes present in toxin of microorganisms, and venom of snakes (Quiros *et al.*, 1992) and insects (Deregnaucourt and Schrevel, 2000). Phospholipases have been known as virulence factors of microbial pathogens (Grant *et al.*, 1997). A lot of work has been performed to establish the structural and functional relationship between the toxin and the cellular membrane.

Phospholipases are classified and designated with the letter A-D according to their hydrolic action on their substrates. The substrates of the phospholipases are phospholipids. Phospholipids are divided into two classes: glycerophospholipids and sphingolipids. The most important phospholipids found in mammalian membrane are phosphatidylcholin (lecithin), phosphatidylethanolamine, and sphingomyelin. Phosphatidylinostol is also present in small amount. Phosphatidylglycerol and

diphosphatidylglycerol (cardiolipin) are also found in bacterial membranes. Cholesterol is another important constituent of the phospholipids (Mollyby, 1978; Dennis *et al.*, 1981; Davidson and Dennis, 1991).

A second group of polar insoluble swelling amphiphiles is associated with the phospholipids in the bilayer membrane by hydrogen bonds and hydrophobic interactions. The hydrolysis of phospholipids by phospholipases is affected by the presence of cholesterol (Mollyby, 1978; Davidson and Dennis, 1991).

Phosphatidate acylhydrolases (phospholipases A) are present in a wide range of cells and they are thought to be essential in the regulation and maintenance of the membrane phospholipids. Phospholipases A are found in bacterial cell wall as well as in mammalian origin. This enzyme is also secreted by exocrine glands such as the pancreas (Mollyby, 1978). Phospholipases A are also found in venom of snake (Quiros *et al.*, 1992), bee (Deregnaucourt and Schrevel, 2000), and spider.

These water- soluble enzymes have specificity towards the 2- acyl position (phospholipase A_2) and calcium ions are essential for their activity (Mollyby, 1978). Most of the phospholipases A have a molecular weight of ~ 14000 except the *Naja naja* (cobra) and the *Apis mellifica* (honeybee) enzymes. The most well known phospholipase A_2 is from porcine pancreases (Vogel *et al.*, 1981). The active form of enzyme consists of 123 amino acids residues whereas zymogene form consists 30 amino acids residues. They are highly stable and have six disulfide bonds. They are not denatured by 8 M urea, 5 M guanidine hydrochloride or heating at 85^{o} C in 2% sodium dodecyl sulphate (SDS) at pH 8.0 (Mollyby, 1978).

On the other hand, intracellular phospholipase A are less defined due to the difficulty in solubilisation and their membrane - bound characteristic. These enzymes have been detected in a large number of bacteria. Phospholipases A can be produced by *Campylobacter coli* (Grant *et al.*, 1997) and *Helicobacter pylori* (Kingma and Egmond, 2002), which may contribute to the cell associated haemolysis.

Escherichia coli is another pathogen which produces phospholipase A and has been

well studied by researchers (Mollyby, 1978; Kingma and Egmond, 2002). This enzyme is present only in outer membrane of bacteria and termed as outer membrane phospholipase A (OMPLA), is an integral membrane enzyme which catalyses the hydrolysis of acylester bonds in phospholipids utilizing calcium as a co-factor (Ubarretxena-Belandia *et al.*, 1999; Deregnaucourt and Schrevel, 2000; Kingma and Egmond, 2002).

2.5.4 Phospholipase A_2

Phospholipases A_2 are important enzymes for the defence and predation of living organisms. They are present in mammals, reptiles, insects, and other invertebrates. They are also found in plants and prokaryotes. However, phospholipase A_2 is strictly isolated from poisonous secretions of snakes (Habermehl, 1981; Takasaki *et al.*, 1987; Davidson and Dennis, 1991) and to some extent in bees (Deregnaucourt and Schrevel, 2000), scorpions, jelly fish, and one species of lizard. The majority of structurally characterized phospholipases A_2 that have been isolated from snake venoms are subject of worldwide interest in explaining the lethal agents of poisonous snakes that human encounter such as cobras and kraits.

Apart from this, there has been medical interest in bee venoms, which may kill hypersensitive individuals by inducing anaphylactic shock rather than haemolytic activity. The main constituent of bee venom, melittin, is reported to have haemolytic activity instead of phospholipases A_2. It is also found to be stimulator to phospholipase A_2. According to Antonio and co - worker, possibly, the harmful effect of bee-venom might be the result of the synergistic action of melittin and phospholipase A_2 (Argiolas and Pisano, 1983). Melittin can be separated from phospholipases A_2 by precipitation with picric acid. However, certain factors such as blood plasma, lecithin, polysaccharides, sulphide, and high concentration of citrate decrease the haemolytic activity of melittin (Habermehl, 1981).

Previously, phospholipases A_2 from prosaic sources have not been studied thoroughly. But now, due to the knowledge of the possible roles of phospholipases A_2 in reproduction, humoral defence, and chronic inflammatory diseases has

increased the interest in the field of phospholipase A_2 research, as those mammalian phospholipases A_2 are thought to have influence in the release of arachidonic acid, the common precursor of prostaglandins (Dennis *et al.*, 1981) and leukotrienes.

Moreover, due to the new evidence of phospholipases A_2 involvement in G protein – linked signal transduction, the effects of phospholipases A_2 in membrane structures has been implicated for other membrane – associated regulatory pathways (Deregnaucourt and Schrevel, 2000).

Phospholipases A_2 catalyse the hydrolysis of *sn*-2 acyl bond of the glycerolphospholipids releasing free fatty acid and lysophospholipids (Dennis *et al.*, 1981; Davidson and Dennis, 1991; Deregnaucourt and Schrevel, 2000). There are various naturally occurring phospholipids which act as substrates for phospholipases these are; phosphatidylcholine (PC), phosphotadylethanolamine (PE), phosphotadylglyserine(PG), phosphatidylnositol(PI), platelet- activatin factor (PAT), plasmalogen, plasmenylcholine, 1- alkylether PC, and others. The substrate can be present in different forms which ranges from mixed micelles of phospholipid, surfactants, and bilayers membrane (Mollyby, 1978; Davidson and Dennis, 1991).

Despite their hydrolytic action on phospholipids, phospholipases A_2 are not considered only as “membrane” enzymes. The extracellular phospholipases A_2 are soluble enzymes that might have quite weak membrane penetrating ability without the help of surfactants or other proteins. Phospholipases A_2 from mammalian pancreases and Indian cobra (N. naja naja) venom require a lipid- water interface for activation and can be further activated by a specific type of phospholipid group, and may utilize the phospholipid activator either for “attaching” to the surface or for stimulation suitable enzyme’s lipid- binding sites that requires catalysis in the surface (Davidson and Dennis, 1991).

Some phospholipases A_2 have product inhibition activity especially by the unsaturated fatty acids. The important point is that the products of phospholipases A_2 hydrolysis of phospholipids are themselves surface active. Partially these products effects may be due to the influence of the products on the phospholipid physical

phase. It has been observed that specificity towards different products demonstrate the potential for physiological regulation via feed back mechanisms (Davidson and Dennis, 1991).

Even though they are associated with catalytic function, phospholipases A_2 have developed a different set of biological functions that are adverse to the pure digestive. Exclusive examples are the venom phospholipases A_2 that have modified as toxins associating with other proteins or by themselves. Phospholipases A_2 are only an example of enzyme among the 20 types of enzymes present in venoms, while in some snake, they are also present in combination with a large number of non-enzyme peptide (Davidson and Dennis, 1991). Toxic phospholipases A_2 can stimulate potent, although poorly defined effects on specific target membranes in animals that have been injected intravenously (IV) or subcutaneously (SC) with the purified enzyme preparations.

Among the most potent phospholipases A_2, single toxic phospholipases A_2 come from sea and Australian snakes. An entirely different type of toxicity is present in the honey-bee venom phospholipases A_2, which is highly antigenic, and capable of inducing anaphylaxis. All the phospholipases A_2 have the basics of their multiple pharmacological effects within the control of one gene. However, some snakes have modified toxic phospholipasesA_2 that associate with other proteins for restoration of their complete toxic potency (Davidson and Dennis, 1991).

Apart from the single chain toxins and toxic protein complexes, one category of "toxic" phospholipases A_2 is noticed which are not toxic individually, but synergistically increase the potency of non - enzyme venom toxins. Phospholipases A_2 from African and Asian cobras (genus Naja) are included in this category. These venoms contain cytotoxic polypeptides 60 – 70 amino acids, which themselves, are thought to have been modified from phospholipases A_2. These cytotoxins are membrane active, having partial specificity for the cells attacked, and work for some time by increasing membrane permeability. The cardio toxins are cytotoxic peptides, which lyse erythrocytes in vitro, block nerve conduction, and can induce contracture, depolarization of skeletal muscle, cardiac fibrillation, and systolic arrest.

Phospholipases A_2 from the Chinese cobra (N. naja atra) and the Mozambique cobra (*N. mossambica mossambica*) has been found to potentiate the effects of cardio toxins. When enzymes and cardio toxins are both injected simultaneously in the laboratory animals, death occurs due to massive haemolysis and from shock, were thought to be enhanced by K^+ released from the lysed cells finally stopping the heart. This entire process can be completed within a few minutes. On the other hand neither the phospholipases A_2 nor cardiotoxin separately can produce the same clinical picture. Other cytotoxins with which phospholipases A_2 synergise are cobra venom direct lytic factor (DLF) and honey bee venom melittin (Vogel *et al.*, 1981; Davidson and Dennis, 1991).

Possibly, the potentiating effects of phospholipases A_2 in cell lysis is due to the in situ production of lysophospholipids, potent biological detergents, the production of which is generally very strictly controlled. This kind of lysis by snake venom phospholipases A_2 acting in synergy with membrane- active toxins is thought to be responsible for the release of several auto pharmacologic agents that are associated with cobra bites, including histamine.

Furthermore, potentiation of bee venom melittin by bee venom phospholipases A_2 can be responsible for the mast- cell degranulation, which causes swelling at the site of bee stings. In laboratory tests, melittin can be replaced by cobra venom DLF or cardio toxins to get synergism with honey - bee phospholipases A_2 in mast cell degranulation .

Therefore, this experiment can show that the underlying mechanism of synergy seems to be partially specific which entails an affinity of the amphipathic cytotoxic peptide for a targeted membrane, and the peptide enhances the effectiveness of the phospholipases A_2 for hydrolysis of the membrane. Though, the individual protein components are interchangeable, no evidence is noticed yet for a direct association of a cytotoxic polypeptide with 1 phospholipases A_2 (Davidson and Dennis, 1991).

Most of the phospholipases A_2 which have been sequenced consist of a single chain of approximately 120- 130 amino acids, with a high content of cysteine. Most snake venoms and the mammalian phospholipases A_2 contain 14 cysteine and the sequences can be classified into one of two major groups after comparison of their cysteine patterns. Bovine and porcine pancreatic phospholipases A_2, have been classified as the archetypal group I enzymes, and *C. atrox* phospholipases A_2, from group II have been crystallized, their X-ray structures determined, and the disulfide-bonding patterns determined (Davidson and Dennis, 1991).

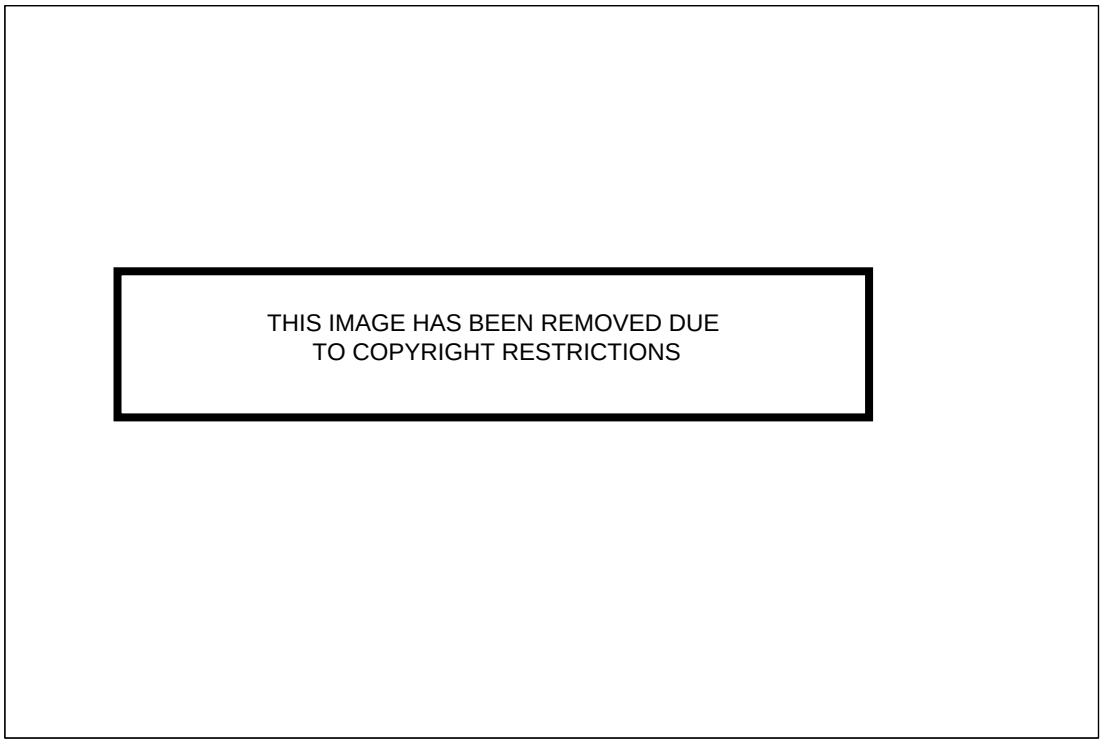


Figure 2.04 Schematic diagram of the suggested action of ceramide on $cPLA_2$ recruitment to the membrane. Reproduced from *The FASEB Journal*. 2001;15:7-9.

2.5.5 Phospholipase C

Phospholipases C (PLC) originally known as lecithinase and referred α- toxin, are the most thoroughly studied phospholipase produced by bacteria (Mollyby, 1978). Phospholipase C has been demonstrated to be an important virulence factor in an increasing number of bacteria such as *Clostridium perfringens* (Mollyby, 1978; Nagahama *et al.*, 1997; Awad *et al.*, 2001; Gomez *et al.*, 2001), *Bacillus cereus* , *L. monocytogenes* (Mollyby, 1978; Nagahama *et al.*, 1997; Beecher and Wong, 2000; Gomez *et al.*, 2001), *P. aeruginosa* (Mollyby, 1978; Gomez *et al.*, 2001), and *Staphylococcus aureus* (Mollyby, 1978).

The most studied cytotoxic effect is the phenomenon of haemolysis. In the beginning, phospholipase C- mediated haemolysis was thought to be quite simple: the phospholipases hydrolyse the phospholipids, which are essential permeability-limiting component . *Clostridium perfringens* (welchii) is the primary causative organism of gas gangrene (Mollyby, 1978; Bryant and Stevens, 1996; Nagahama *et*

al., 1997; Awad *et al.*, 2001).

Gas gangrene or clostridial myonecrosis is characterized by a rapid spread of tissue necrosis combined with a lack of leukocyte infiltration at the site of infection (Awad *et al.*, 2001) and death in approximately 30 % of patients. Alpha- toxin (phospholipase C) has been reported to possess various biological activities, such as haemolysis, lethality, necrosis, contraction of blood vessels and ileum, and platelet aggregation (Sakurai *et al.*, 1993; Nagahama *et al.*, 1997). Phospholipase C hydrolyses both phosphatidylcholine and sphingomyelin which are important constituents of eukaryotic cell membranes (Nagahama *et al.*, 1997; Awad *et al.*, 2001).

Candida albicans is an opportunistic pathogen causing infections in immunocompromised patients. The organism is a dimorphic fungus, which can change between two phases: yeast and hyphal phases, depending on the growth conditions. *Candida* species have the ability to produce a variety of hydrolytic enzymes, such as proteases, lipases, phosphatases, esterases, and phospholipases. They are known to mediate candidal pathogenesis by facilitating the hyphal invasion in disseminated candidiasis (Luo *et al.*, 2001). Adhesions, dimorphism and the secretion of specific hydrolytic enzymes have been thought to be possible virulence factors. *Candida albicans* secrets a haemolytic factor which causes the release of haemoglobin and use the iron, but the haemolytic factor was unable to be identified (Watanabe *et al.*, 1999). They demonstrated the haemolytic factor in the culture supernatant of *C. albicans*.

2.6 Underlying Haemolytic Disorders Predisposing to Infections

There are certain conditions in which haemolysis occurs and predispose to bacterial infections. Various specific reasons are suggested for the different underlying haemolytic diseases predispose to infection. However, two possible mechanisms are thought to be responsible: reticuloendothelial blockade and presence of increased haemoglobins or iron for microbial growth (Berkowitz, 1991).

It has been shown that in the experiment carried out in mice, infusion of sensitized RBCs enhance the fatality rate of *Salmonella typhimurium* infection. Similarly, infusion of RBC stroma causes a decrease in the clearance function mediated by the hepatic macrophages complement receptor, and increases the fatality rate for pneumococcal infection (Berkowitz, 1991).

It has been proven *in vitro* that macrophages exposed to sensitized RBCs demonstrate decreased rates of bacterial killing. It is suggested that ingestion of these RBCs by the reticuloendothelial system may interfere with other clearance function of the reticuloendothelial system, but no decrease in reticuloendothelial clearance activity could be demonstrated in studies of humans with thalassemia (Berkowitz, 1991).

According to some studies, iron therapy given for iron deficiency can lead to an increased occurrence of severe infections, especially in the case of enteric bacilli. It is demonstrated that blood enhances the development on an infection in an enclosed space such as the peritoneal cavity by supplying iron for microbial growth (Berkowitz, 1991).

It is still undefined if haemolytic conditions predispose to infection by providing iron to microorganisms. However, it is known that repeated blood transfusions leading to iron overload in combination with the use of deferoxamine, and iron chelator, predispose to severe infections. This property of iron chelator has been demonstrated in experimental animals with *S. typhimurium* infection and in human with thalassemia with *Yersinia enterocolitica* infection. *Yersinia enterocolitica* does not produce siderophores itself, but they can assimilate iron by utilizing the siderophores produced by the other bacteria when they exist together in the colon. Deferoxamine is a derivative of siderophores from *Steroptomyces pilosis* which is used to treat the iron overload individual. *Yersenia enterocolitica* present in the blood circulation utilise deferoxamine as its siderophores and can multiply. Deferoxamine also seems to predispose patients who are on therapy for iron or aluminium overload to infection with zygomycetes (Berkowitz, 1991).

Sickle cell disease is the most common haemolytic disease predisposing to infection. The hyposplenism is primary cause of this predisposition, which follows the development of micro infarction of the spleen, but a defect in C3b fixation to bacterial surface also is involved. Hyposplenism can be found in children with sickle cell disease who are as young as four months old and is usually detected by the age of one year. This defective host defence predisposes these individuals to infections with encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (Rodak, 2002). *Escherichia coli* and salmonella are also common causes of bacteremia in Saudi Arabia and tropical Africa. Another fatal infection in tropical Africa in these individuals with sickle cell disease is malaria. Due to the high incidence of G6PD deficiency in- patient with sickle cell disease, it is thought that the G6PD deficiency had a protective effect on these individuals.

Individuals with sickle cell disease are susceptible to osteomyelytis caused by non-typhoid salmonellae. The following series of events was noted to produce the infection.

I. Poor sanitation was the cause of frequent gastrointestinal infections with salmonellae.
II. Bowel ischaemia facilitates haematogenous spread of these organisms.
III The host defences against salmonellae might be compromised by hepatic dysfunction and haemolysis.
IV Bone infarction predispose to osteomyelytis.

Both clinical and epidemiologic evidence indicates that G6PD deficiency is cause of haemolytic predisposition to infection. G6PD deficiency affects the RBC, causing haemolysis. The enzyme is essential for the production of oxidative metabolites, H_2O_2 required for bacterial killing by phagocytes. G6PD deficiency also seems to predispose to a particularly severe clinical manifestation in patients with rickettsial infections (Berkowitz, 1991).

2.7 Predisposition to Infection by Therapy for Haemolytic Disorders

There are various types of inherited and acquired haemolytic diseases. However, limited variety of therapy is used for the treatment of these disorders. Despite their significance in treatment, they predispose to certain infections (Berkowitz, 1991).

2.7.1 Blood transfusion

Blood transfusion is the most commonly used treatment for anaemia but it is also a very convenient route for transmission of infections. There are a large number of infections, which can be transmitted by blood transfusion. Viral agents cause hepatitis (non-A, non- B and hepatitis B) and cytomegalovirus and HIV. Malaria is another disease that is transmitted by transfusion in malarial endemic areas. *Trypanosoma cruzi* is can be transmitted through transfusion in its endemic region. Moreover, a large number of bacterial infections were reported to be transmitted by transfusion of contaminated blood, which might have occurred during storage. A list of infectious agents that are transmitted by blood transfusion are shown in the following table (Berkowitz, 1991).

Table 2.06 Infectious agents transmitted in blood

Group	Specific agents
Viruses	Hepatitis B, hepatitis non- A non- B, cytomegalovirus; HIV; Epstein- Barr virus; HLTV- 1
Rickettsias	*Rickettsia prowazeki*
Bacteria	*Treponema pallidum*, *Brucella*, *Y. enterocolitica*;
Protozoa	*Plasmodium spp*; *Trypanosoma cruzi*; *Trypanosoma brucei*; *Babesia* species; *Toxoplasma gondii*
Nematodes	Filaria

2.7.2 Immunosuppression

Corticosteroids are used for immunosuppression basically for the treatment of autoimmune haemolytic anaemia. High doses for prolonged periods can predispose to the infectious complications of chronic immunosuppression (Berkowitz, 1991).

2.7.3 Splenectomy

Splenectomy is common remedy for patients with hereditary haemolytic anaemias such as hereditary spherocytosis and thalassemia major. After splenectomy these patients are susceptible to bacteremia with encapsulated organisms such as *S. pneumoniae*, *H. influenzae*, and *Neisseria meningitidis*.

2.8 Zieve's Syndrome

Alcohol associated haemolysis in Zieve's syndrome also has raised interest in researchers. Even though a lot work has been carried out in this subject, no one is able to identify the relationship between the various components of the syndrome and actual cause of the transient haemolysis (Melrose, 1990). Alcoholism and alcohol related liver disease is a major health problem affecting millions of people per year worldwide. The important point to remember is that alcohol related liver disease is quite clinically asymptomatic so people are less aware of this problem. Recently a study showed that healthy subjects might have slightly elevated transaminase in routine physicals, blood donor screening. In peripheral blood smears we can observe red cells those are more spherical, that is cell lacking the central pallor.

2.8.1 Mechanisms of haemolysis in Zieve's syndrome

The proper mechanism of alcohol related damage is still a mystery. It is proved that the old concept of nutritional deficiency of primary importance is incorrect. Experiments and clinical evidence has revealed that significant alcohol related damage does occur despite maintenance of adequate nutrition. Most of the studies

have centered on the possibility that some product of the metabolism of alcohol might be responsible for the disease. Acetaldehyde is thought to be major metabolite for the damage. However, individual differences in the metabolic pathways should be considered for the observation that not all alcoholics develop significant liver damage.

Various chemical agents and drugs have been found that are thought to cause haemolysis by oxidative denaturation of haemoglobin resulting the production of methaemoglobin, sulfhaemoglobin, and Heinz bodies. Drugs such as naphthalone (mothballs) and dapson (for leprosy) are example of this agent (McKenzie, 1996; Rodak, 2002). Moreover, water also has haemolytic properties. When water enters the vascular system during transutheral resection and it produces haemoglobinemia and haemoglobinuria due to the osmotic lysis of red blood cells (McKenzie, 1996).

2.9 Sago Haemolytic Disease

Sago palm (*Metroxylon sagu*) is an important part of diet in some parts of (PNG). Sago haemolytic disease is an acute intravascular haemolytic anaemia, which is thought to have some association with the ingestion of stale sago. The occurrence of this disease seems to be high during food shortage, particularly in remote rural areas (Donovan *et al.*, 1977).

Figure 2.05 *Metroxylon sagu*

Clinical features of this manifestation are severe anaemia and jaundice of sudden onset with very darkish urine called “dark red” or “port wine” in colour (Donovan *et al.*, 1977). Fever, headache, vomiting, lethargy, dehydration, and hepatosplenomegaly are other important clinical findings (Taufa, 1974). As the condition deteriorates, irrational, uncontrollable behaviour, grinding teeth, and later unconsciousness are commonly observed.

The studies performed to investigate the aetiological agents of sago haemolytic disease have isolated several bacteria and fungi from sago grains. However, they all were not considered to be pathogenic and responsible for this crisis. *Candida valida, B. cereus, Paecilomyces lilacinus,* and *Tilletiopsis minor* were reported be either non-pathogenic or contaminants (Donovan *et al.*, 1977).

Apart from this, some researchers have studied a number of mycotoxins including aflatoxin, ochtratoxin, F_2 toxin and byssochlamic acid but the result was negative.

Furthermore, other researchers screened samples for mycotoxin, aflatoxin, zearalenone, orchatoxin, sterigmatocyn and citrinin, but they were not found in the sample. Some experiments were carried on saponin which has a haemolytic property but it didn't help much because saponin is not present in sago and it is not toxic to human if it taken orally.

According to the paper, due to certain circumstances, confirmatory laboratory tests were not possible to perform. However, it was able to suggest that the haemolysis was acute and intravascular (Donovan *et al.*, 1977).

Some work has suggested that the occurrence of this disease may have a similar mechanism as in "favism", G6PD deficient individuals are at more risk of haemolysis after consumption of fava bean. It is reported that the G6PD deficiency is relatively common in those area where there was outbreak of sago haemolytic disease. Therefore, it is suggestive that there is possibility of the association of both genetic disorder and some other environmental agents (Donovan *et al.*, 1977).

It is obvious that, very little about the disease is known. However, a lot of work is ongoing to determine the causes and the extent of the health risks to consumers. Some common food pathogens such as *E. coli, S. aureus, B. cereus, L. monocytogenes* and *Salmonella spp,* and yeast and mould are most suspected and samples are under investigation for their presences. These works will be definitely significant for the identification of mycotoxin and better knowledge of the disease.

2.10 Melanesian Ovalocytosis

Melanesian ovalocytosis is an hereditary, clinically asymptomatic trait characterised by oval shaped red blood cells (Kimura *et al.*, 2003), which is commonly found in coastal parts of PNG and Malay Archipelago up to 35 to 40 % (Tanner *et al.*, 1991; Liu *et al.*, 1994; Bruce *et al.*, 2000). Many studies have shown that the basic molecular defect in MO is the deletion of nine codons in the erythroid band 3 (Tanner *et al.*, 1991; Sarabia *et al.*, 1993; Liu *et al.*, 1994; Bruce *et al.*, 2000).

The toxins produced by some of those organisms isolated from sago are found to be haemolytic. Moreover, there are several reported cases of sago haemolytic disease (Taufa, 1974; Donovan *et al.*, 1977). Even though, the definite cause of sago haemolytic disease is not yet identified, however, this project is trying to establish that MO could be predisposing factor for sago haemolytic disease in people of PNG.

2.11 Conclusion

Numerous, never-ending efforts of researchers are still undergoing for the investigation of the premature haemolysis and its aetiology. There is still a huge amount of unsolved, unidentified mysteries regarding the association between haemolysis and infections. It is already reported that there are wide ranges of factors, which have direct or indirect effects for haemolysis. It can be either inherited or acquired. It can be mechanical or physical too. A lot of work has been carried out for the identification of those causes as well as development of various types of laboratory techniques for the detection and determination of those factors.

Very useful classifications and categorisations are well documented which have great help for clinician to diagnose the infectious disease with haemolysis and manage the patients. Similarly, it has equal importance for laboratory investigations. There are so many influencing factors for the haemolytic conditions associated with infections that only very careful physical examination and detailed history taking will lead to fruitful result. Some information is extremely important and useful for the identification and diagnosis of the disease. Family and personal history such as genetic defects and drug addictions; epidemiological history such as geographical and travel history; medical history; and a careful and appropriate laboratory investigations might be able to save the life of the patient.

CHAPTER 3 - GENERAL MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Finger prick

The participation of individuals and their blood taking was approved by the JCU Human ethics Committee and granted the approval number H1835. Blood from participants was obtained by finger prick using lancets fitted in lancing device (Accu-chek, Roche Daignostics). Blood smears were immediately prepared after finger prick and left for air dry. Dried blood smears were fixed in methanol. Fixed blood smears were then stained using Harleco's diff quik stain set 64851, which is a modification of the Wright's stain technique and mounted after air drying. They were examined under oil immersion lense. Two out of 35 samples were identified to be ovalocytosis based on standard methods (Rodak, 2002) and these patients were recruited into the study with their kind permission.

Figure 3.01 Capillary blood collection

3.1.2 Venous blood draw

Venous blood was drawn in lithium vacutainer from the two participants who were identified as ovalocytic in initial screening, and ghost cell was prepared from the fresh blood sample.

3.2 Haemolysin from Bacteria, Fungi and Yeasts

The organisms used in this experiment were all isolated and identified from sago and screened for primary haemolytic activity by Andrew Greenhill, they were provided with his kind permission. Microorganisms included in this study were *C. perfingens*, *Penicillium steckii* and an unidentified haemolytic yeast.

Figure 3.02 Stale sago starch derived from *Metroxylon sagu*

3.2.1 Isolation of bacterial toxin

3.2.1.1 Preparation of dialysis tube

For the removal of glycerine, the required length of dialysis tube (Sigma) was taken and washed under running water for 4 hrs. The sulphur compound was removed by treating the tubing with a 0.3% (w/v) solution of sodium sulphide (BDH) at 80ºC for 1 minute. The tubing was rinsed with hot water (60ºC) for 2 minutes, and acidified with a 0.2% (v/v) solution of sulphuric acid, followed by rinsing with hot water for the removal of acid. The tubing used had the capacity of retaining most proteins of mol. wt. of 12,000 or greater.

3.2.1.2 Preparation of culture medium for bacteria

For the preparation of this fermentor medium, 25 g of protease peptone (oxoid) and 6 g of yeast extract (oxoid) were dissolved in 150 ml of deionized water and filled in treated dialysis tube and dialyzed for 3 days in cold against 1 litre of distilled water. A mass of 2 g of cysteine hydrochloride (Sigma), 10 g of $NaHCO_3$ (Sigma), 20 g of dextrin (sigma), 10 g of acid- hydrolysed casein (casamino acids), (Difco, USA) and 2 g of KH_2PO_4 (BDH) was added as supplements to the dialysate and pH was adjusted to 6.7 with 3 N NaOH. Then the dialysate was sterilized at 115ºC for 20 min.

3.2.1.3 Culture

The bacterial strain was inoculated into 20 ml of TGY medium (Appendix 1) and incubated for overnight at 37ºC. Then this culture was seeded to fermentor medium (Appendix1).

Oxygen from fermentor medium was removed by supplying high purity nitrogen compressed (UN No. 1066, Gas Code 034, Size G, NSW) gas. 500 ml of culture growth of TGY medium was inoculated into the fermentor medium. The culture was put in the anaerobic jar and again it was treated with nitrogen to make the jar devoid

of oxygen. The jar was incubated overnight at 37ºC.

3.2.1.4 Toxin extraction

Cholesterol acetate solution (Sigma) was added slowly into the culture growth to precipitate the theta toxin. Benzamidine hydrochloride hydrate, 2 g in 1 litre culture growth ;12 mM (Sigma) was added at the same time to prevent alpha toxin degradations by proteases. Two centrifugation at 10,000 *g* at 4ºC (suprafuge 22, Heraeus, Germany) for 20 min were done to remove the bacteria and the aggregation of cholesterol with theta toxin.

The supernatant was separated from the deposit and ammonium sulfate (BDH), 317g/l (approx.60% saturation) was added to this cell-free culture medium and was kept overnight at 4ºC. Then the culture medium was centrifuged at 16,000 *g* for 15 min. The supernatant was discarded and the precipitate was dissolved in 20 ml of buffer A and centrifuged at 16,000 *g* at 4ºC for 15 min in order to eliminate the insoluble residue. The supernatant separated and was kept in freeze drier (Dynamic freeze drier) overnight. The dry residue was dissolved with 10 ml of ethanol and finally it was diluted with 40 ml of PBS. It is stated that alpha toxin's activity is stable for several months if it is kept at -20ºC with addition of 5% glycerol.

3.2.2 Fungal culture and isolation

3.2.2.1 Media preparation and fungal culture

Wheat and rice were used as culture media for the *P. steckii* isolated from sago, because they are commonly infected by fungus (Moreau, 1979). An aliquot of 25 g of each grain was measured and mixed with 10 ml of deionized water. Then the media were sterilized and 5 ml of deionized water was added before mould inoculation. The mould was inoculated in the media and incubated at 30ºC for approximately 4 weeks. The culture growth was gently shaken at regular intervals.

3.2.2.2 Toxin extraction

The substrate and fungal biomass was blended in Waring blender (JM Scientific Instruments, Australia) in a Fume Cabinet (BMS 022). The blended samples were stored at −20°C in plastic zip-lock bag till further process. Approximately 8 g of sample was mixed in 40 ml TDS (toxin dilution solution). The beaker-containing sample was kept in esky of ice for 10 min for cooling. Then it was sonicated for 2 min, using sonicator (Biosonik III, Brownhill Sceintific, N.Y.) in PC3 lab. After resting on ice for 5 min it was re-sonicated. During sonication, ear protection was used and no one was present in that room.

The beaker was placed in an esky of ice while being sonicated to prevent it getting too hot. The sonicated sample was put in 50 ml centrifuge tube and centrifuged at 5000 rpm for 20 min. The supernatant was filtered using Whatman No. 4 filter paper. Filtered supernatant was collected in a clean centrifuge tube and re-centrifuged, this procedure was repeated. A volume of 40 ml TDS /ethanol was added to the residue from the original tube and mixed well. It was centrifuged at approximately 5000 rpm for 20 min. Supernatant was filtered using Whatman No. 4 filter paper.

Some aliquots of the extracts were stored in fridge for short-term storage (1-2 days), and some of them were stored for longer period in freezer.

3.2.3 Culture and toxin extraction of haemolytic yeast

3.2.3.1 Culture of haemolytic yeast

Yeast was sub-cultured on Carrot potato agar (CPA) media and incubated at 30ºC for two days. This yeast was again sub-cultured on Sabouraud dextrose agar slope and incubated at 30ºC for 2 weeks. Finally, the yeast was inoculated on blood agar and sago D and sago S agar plates. After 2 weeks incubation, the toxin extraction procedure was performed.

3.2.3.2 Toxin extraction

Material of two agar plates was placed in a stomacher bag and 100 ml of TDS was added and stomached in laboratory blender (Stomacher, John Morris Scientific) for 1 min. The blended material was sonicated applying the same method for fungal toxin.

3.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis by Laemmli Discontinuous Buffer System

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used, simple and reliable method (Maguire *et al.*, 1989) for rapid protein separation (Baines, 2001). In this system, migration of protein is not only determined by intrinsic electric charge of polypeptides but by the molecular weight. Sodium dodecyl sulphate (SDS), which is an ionic detergent, has various actions on the protein molecules. It denatures proteins by coating the hydrophobic tail of the polypeptide structure, and it also damages the hydrogen bonds, obstructs the hydrophobic interactions, denatures the protein molecules, and eventually minimizes the differences in molecular forms by destroying the secondary and tertiary structures.

The complete denaturation of proteins is achieved by heating them in buffer containing SDS and a thiol reducing agent such as diothiothreitol (DDT). After disruption of disulfide bonds by DDT, polypeptides change to a flexible rod – shaped and possess an uniform negative charge per unit length.

Utilizing the gel electrophoresis technique, a complex protein mixture can be separated into numerous bands on a gel which can be easily visualized by appropriate staining technique and estimated by densitometer (Bio-Rad instruction manual. Mini-Protean 3 cell).

Discontinuous SDS-PAGE was performed according to the method described by Laemmli, using a Bio-Rad Mini Protean 3 cell gel apparatus for the separation of

RBC membrane (ghost cell) protein fractions as well as to examine the effect of certain haemolytic toxins on the RBC membrane proteins. Two gels, stacking gel of 4 % and resolving gel of 12 % was prepared according to the formulation provided (Appendix 2). All the apparatus were assembled and buffers and reagents were also prepared following the protocol provided.

Figure 3.03 Screening blood slides

CHAPTER 4 - DETERMINATION OF HAEMOLYTIC ACTIVITY OF ORGANISMS IMPLICATED IN SAGO HAEMOLYTIC DISEASE

4.1 Introduction

Initial screening for haemolytic activity of SHD suspected organisms was undertaken by qualitative demonstration of zones of haemolysis on blood agar, a more quantitative method was required to further establish the ability of the selected organism's toxin to lyse red blood cells under certain incubation conditions. This assay was used to help determine the optimal conditions for cultivation of these organisms and extraction of toxin, in terms of haemolytic activity. This information will aid in maximising toxin production so work planned for Chapter 5 may be conducted. The haemolytic activity of the selected organisms (Chapter 3), plus *C. perfringens* ATCC 13124 used as a control, was demonstrated by methods based on Bernheimer (Bernheimer, 1988).

4.2 Materials and Methods

4.2.1 Erythrocyte suspension preparation

In a polypropylene centrifuge tube of 50 ml capacity, 2 ml of lithium-heparinized blood was diluted in 40 ml of 10 mM PBS (pH 7.2) or 10 mM Tris buffer and centrifuged at 3000 *g* (Eppendorf, centrifuge 5804, Germany) for 5 min. The supernatant was discarded, and the sedimented red cells were re-washed with 20 volume of PBS. Finally, 2 ml of the sedimented erythrocytes was diluted in 1.5 ml of PBS.

4.2.2 Haemolytic assay

Fungal, bacterial and yeast isolates were cultivated on various media and toxins extracted as per Chapter 3. This was undertaken to maximise the ability to detect and extract toxins, as it has been found that organisms express toxins under varying conditions (Mollyby, 1978; Betina, 1984b; Bernheimer, 1988; Manns *et al.*, 1994; Luo *et al.*, 2001)

In a clean and dry glass tube, 350 µl of erythrocyte suspension was added to 2.65 ml of toxin extract and incubated at 37ºC at 150 rpm in shaker (Shaker Bioline Eoo3523, Edwards Instrument, Australia) in the presence of light source for 30 min. Exactly after 30 min, 400 µl sample from that erythrocyte and toxin mixture was transferred to micro centrifuge tube and kept in fridge for 10 min. Then, the sample was centrifuged at 120 *g* (Eppendorf, centrifuge 5415D, Germany) for 7 min and 100 µl of the supernatant was placed in microplate and absorbance was read at 540 nm using spectrophotometer (Labsystems Multiskan EX, Finland). The procedure was repeated at 1, 2, 4, 6 and 8 hours.

All assays were performed with the same condition, that is, on normal human erythrocytes at 37°C for 30 min, 1 hr, 2 hrs, 4 hrs. incubation for *C. perfringens* and *P. steckii* toxin extracted with TDS/ ethanol. Other assays were carried on for 6 hrs and 8 hrs incubation. The percent haemolysis is presented in comparison to 100% haemolysis of erythrocytes in PBS with 10% Triton X-100, a substance that causes complete lysis of red cells and release of haemoglobin . The values are the means ± standard deviations (error bars) of triplicates.

4.3 Results

Varying comparisons of cultivation media and extraction conditions were tested for relative haemolytic activity. *Penicillin stecki* grown on wheat and rice and extracted with TDS/Ethanol or TDS alone were compared (Chapter 3). The haemolytic *Candidia* spp was grown on two types of sago based media (Sago D and S, Chapter 3) and BA and products were extracted with TDS/Ethanol and TDS alone. *Clostridium perfringens* was included as a control as its haemolytic activity is known.

Firstly, extract from *C. perfringens* isolated from sago and *C. perfringens* ATCC 13124 was analyzed. The result demonstrated that percent haemolysis was greater in *C. perfringens* ATCC 13124 than *C. perfringens* isolated from sago. *Clostridum perfringens* isolated from sago demonstrated minimal or negligible haemolytic activity (Figure 4.01).

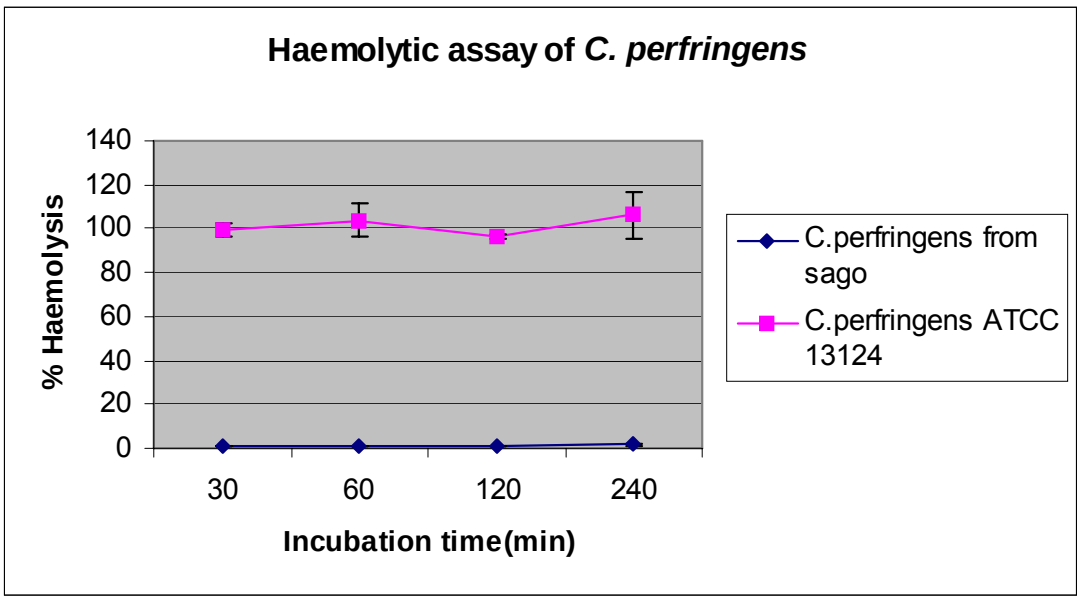


Figure 4.01 Haemolytic activities of *C. perfringens* toxin extracted from sago (◆) and *C. perfringens* ATCC 13124 (■)

Penicillium steckii toxins extracted with two separate methods have different levels of haemolytic activity. The experiment where *Penicillium steckii* toxin was extracted with TDS demonstrated higher haemolytic activity, when analysed, than when the toxin was extracted with TDS/Ethanol. (Figure 4.03). Also, the cultivation media most likely to make the toxin available is wheat (percent haemolytic 80% at 240 min) compared to rice (percent haemolytic 46% at 480 min) (Figures 4.02 and 4.03)

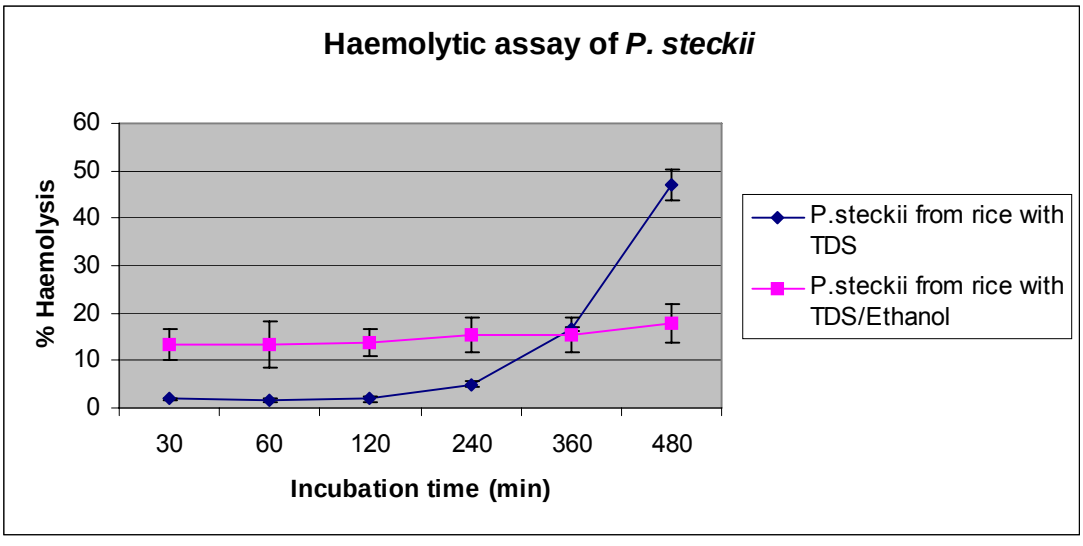


Figure 4.02 Haemolytic activities of toxins extracted from *P. steckii* with TDS (◆) and with TDS / Ethanol (■) grown in rice medium.

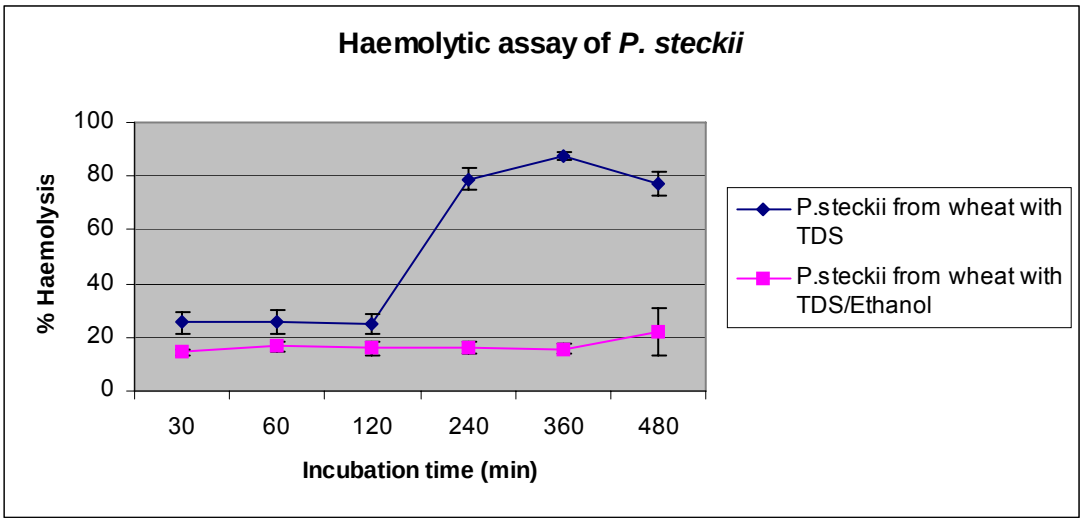


Figure 4.03 Haemolytic activities of toxins extracted from *P. steckii* with TDS(◆) and with TDS / Ethanol (■) grown in wheat medium

Similar to *P. steckii*, the toxin extracted from *Candida* spp is generally more active when extracted without ethanol (Figure 4.04 and 4.05). The media where the *Candida* spp was cultivated also made a difference, in that the activity of the extract taken from *Candida* spp on BA was more active than those cultivated on sago-based media.

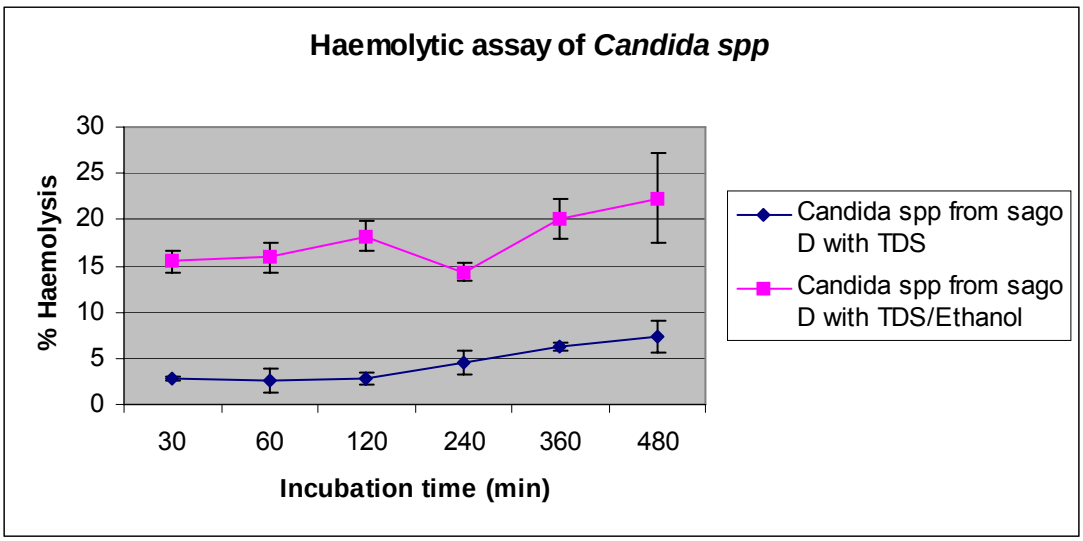


Figure 4.04 Haemolytic activities of toxins extracted from *Candida spp* with TDS (◆) and with TDS / Ethanol (■) grown in sago D

The decline phase (Figure 4.05) could have been caused by the unavailability of fresh RBC supply.

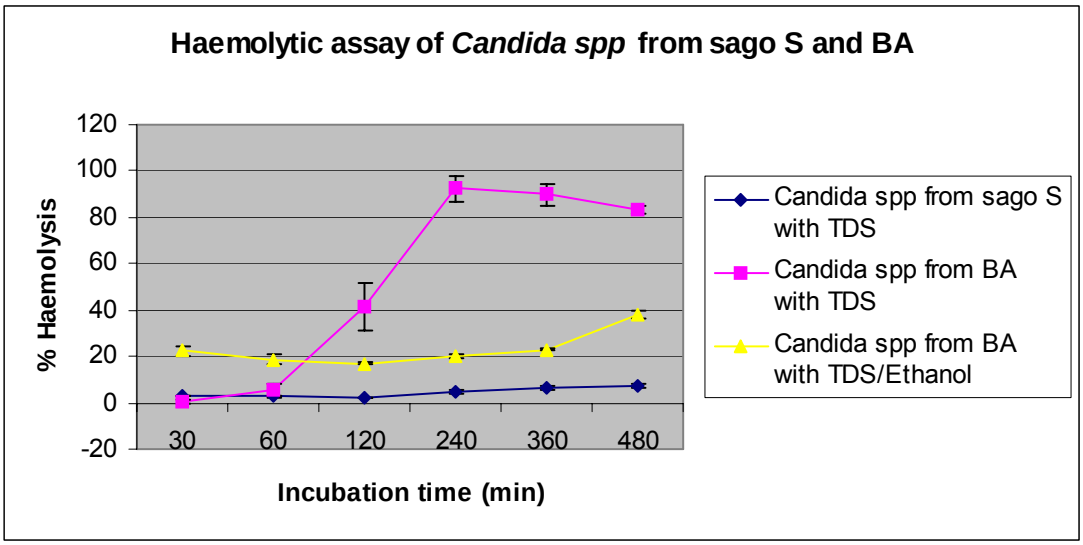


Figure 4.05 Haemolytic activities of toxins extracted with TDS from *Candida spp* grown on sago S (◆), and grown on BA (■), and extracted with TDS / Ethanol (▲) grown on BA.

4.4 Discussion

Various haemolysins are produced by different organisms (Mollyby, 1978) and not all haemolysins have similar haemolytic properties. In this experiment various extraction and incubation conditions of SHD implicated micro-organisms were tested for haemolytic activity using a technique that could semi quantify the extent of the haemolysis and there make comparisons possible.

Clostridium perfringens from the food lab collection (ATCC 13124) and a strain isolated from sago was tested (Figure 4.01) . The organism from the collection appeared more haemolytic than the *C. perfringens* isolated from the sago. *Clostridium perfringens* produces alpha - toxin, phospholipase C (a lecithinase) (Sakurai *et al.*, 1993; Nagahama *et al.*, 1997) of molecular weight 43 kDa (Mollyby, 1978). It is reported that the phospholipase C hydrolyzes phosphatidylcholine present in the outer layer of erythrocyte membrane, to phosphorylcholine and 1,2-diacylglcerol (Mollyby, 1978; Sakurai *et al.*, 1993; Nagahama *et al.*, 1997). It is capable of splitting sphingomyelin and lysophosphatidylcholine. Once the membrane structure is disrupted, cell lysis will be followed by haemolysis. The fact that the species from sago was not able to produce detectable haemolysis indicates that this organism's presence is unlikely to be clinically relevant in terms of SHD. The

analysis of more isolates would need to be carried out to determine this.

Candida spp showed lesser or minimum haemolytic activity depending on the growing media and extracting solution. After observing the result, it is obvious that toxin extracted with TDS from an organism grown on BA demonstrated stronger haemolysis than the toxin extracted with ethanol and using other incubation conditions.

Candida spp produces a wide range of hydrolytic enzymes, which are phospholipases, phosphotases, proteases, lipases and esterases (Luo *et al.*, 2001). Proteases and phospholipases are hydrolzing enzymes that enhance the invasion of candida (Fallon *et al.*, 1997).

Some studies have shown that the *Candida* spp are also capable of producing haemolytic factors (Manns *et al.*, 1994; Tanaka *et al.*, 1997; Luo *et al.*, 2001). Haemolytic factors produced by *C. albicans* lyse the erythrocytes and release haemoglobin. Then *C. albicans* utilize iron derived from haemoglobin and increase the pathogenicity of the organism (Manns *et al.*, 1994; Tanaka *et al.*, 1997; Watanabe *et al.*, 1999). According to the study, it has been proven that the haemolytic activity is influenced by the incorporation of glucose in the growing media (Watanabe *et al.*, 1999; Luo *et al.*, 2001).

One noticeable factor is that after 6 hours incubation, the haemolytic activity has started to decline. It is suggestive that, this could have occurred due to the complete lysis of red blood cells presented in that suspension.

Penicillium steckii toxin produced in wheat media demonstrated higher haemolytic activity than the toxin produced by *P. steckii* grown on rice media, though, little is known about the haemolytic mechanism. It is reported that *P. steckii* produces carboxylic acids as tanzawaic acid E and and F, an unidentified 3,7-dimethyl-1-8-dihydroxy-6-methoxy-isochroman and a identified mycotoxin 3,7-dimethyl-1- 8-dihydroxy-6-methoxy-isochroman (Betina, 1984b; Malmstrom *et al.*, 2000).

Some workers has reported that *P. steckii* is closely related to *P. citrinin* and

P. sizovae (Malmstrom *et al.*, 2000) while other workers have suggested *P. steckii* and *P. citrinin* are synonyms. Citrinin produced by *Penicillium spp* has shown to have inhibitory effects on cholesterol and triglyceride biosynthesis in liver of rat (Betina, 1984a).

The results of this series of experiments supports previous observations that the extent of haemolytic activity an organism may produce depends on extraction method and incubation media / conditions. These data suggest that the haemolytic fungi and yeast initially screened and identified as potential candidates as aetiological agents for SHD may be correct as they both, upon analysis, were shown to produce haemolytic compounds with extensive activity when the optimal conditions were used. *Clostridium perfringens* clearly possesses the potential to produce clinical significant haemolytic compounds but the isolates from sago failed to demonstrate this. Further study with more isolates from sago would need to be conducted to determine if these findings are significant.

It is clear that if the haemolytic activity of future *P. steckii* isolates is required to be analysed, incubation with wheat and extraction with TDS are the preferred condition. Similarly the haemolytic fraction from *Candida* spp is more readily made available with the use of TDS. Interestingly, BA rather that media prepared from sago potentiated the fraction's expression. This may not be surprising in that the presence of red cells may initiate the expression of the haemolysin. Regardless, this media seems the preferred media for the extracting haemolysins from *Candida* spp.

The fact that the haemolytic fractions from both *P. steckii* and *Candida* spp are available using TDS as a solvent may aid in identifying the compound's composition. Further chemical analysis would be required.

CHAPTER 5 - THE ACTIVITY OF HAEMOLYTIC FRACTIONS ON RED CELL MEMBRANES FROM NORMAL AND MELANESIAN OVALOCYTOSIS RED CELLS

5.1 Introduction

The cultivation and extraction conditions of the selected organisms determined to be optimal in Chapter 4, were included for this study. These extracts were exposed to red cell membranes to help in determining the activity of the extracted fractions. To further the study, red cells membranes were freshly prepared from both Melanesian Ovalocytic (MO) cases (heterzygote band 3 delection, Chapter 2) who were identified by initial screening (Chapter 3), and one normal person for control. The extract activity could then be compared to determine firstly if the target of haemolysis are red cell membrane proteins and secondly, if MO affected cells are more susceptible to lysis. The later would help determine if MO potentiates haemolysis by these toxins and therefore if MO could be a host factor that predisposes SHD in PNG.

Different proteins present in human erythrocyte membranes can be separated and demonstrated in different bands of specific molecular weight with the implementation of gel electrophoresis. Bio-Rad Mini Protean 3 cell slab gel was used in this experiment following the Laemmli discontinuous SDS-PAGE system because it is very popular and useful tool for separation of proteins of erythrocyte membrane.

5.2 Materials and Methods

5.2.1 Preparation of erythrocyte membranes (ghosts cells)

Erythrocyte membrane ghosts were prepared by the method of Dodge et al (Dodge *et al.*, 1963; Jarolim *et al.*, 1991; Yoshida *et al.*, 1991). Blood was drawn from the antecubital veins of the participants and lithium- heparinized 5 ml of whole blood was centrifuged at 1,500 x *g* for 10 min, and buffy coat was aspirated along with plasma. The packed red cells were washed 3 times with 10 ml of 5 mmol/l phosphate buffered saline (PBS), pH 8.0, and were suspended finally in an equal volume of this

isotonic phosphate buffer.

A volume of 1 ml of the suspension was pipetted into 10 ml of polypropylene centrifuge tube, and centrifuged as before. The sedimented red blood cells were lysed by mixing in 10 ml of cold 7 mmol/l sodium phosphate buffer, pH 7.4, and kept at 4ºC for 20 min. The suspension was centrifuged at 14,000 x *g* (Eppendorf 5804R, Germany) for 20 min at 4ºC. After centrifugation, the supernatant was aspirated without loss of ghosts. The ghosts were washed continuously with the lysis buffer up to 6 times, and finally, suspended in 2 ml of the lysis buffer. The viscous button of granulocyte debris was removed carefully, because it contains protease activity (Fairbanks *et al.*, 1971) . Aliquots were stored at -20ºC until used (Yoshida *et al.*, 1991).

5.2.2 Preparation of test samples

An equal volume of ghost erythrocytes and toxins was mixed and incubated at 37°C for 1, 6 and 12 hours. After treatment with toxin the sample was treated with 2× treatment buffer (0.125 M Tris-Cl, 4%v/v glycerol, 0.2 M DDT, 0.02% bromophenol blue) in Eppendorf tube and placed in boiling-water bath (at 95°C) for 5 min. The lid of the microtube was pricked in 3 or 4 places with fine needle to avoid the explosion of samples during boiling. The treated sample was kept in ice before it was loaded in the gel. The treated sample can be stored at -20°C up to 6 months.

5.2.3 Sample loading

A volume of 20 µl of sample was loaded very gently with a micropipette (Labysystems, Finnpipette) using gel loading tips beneath the buffer in each well. A normal sample and bovine serum albumin (BSA, Sigma)were also run with the test samples as controls. All the wells should be loaded with equal volume of the sample. The extra wells are also loaded with sample buffer without the sample to prevent the spread of sample laterally from adjacent well during electrophoresis.

After loading the samples, the lid was placed on the Mini Tank making sure that the suitable power supply was oriented with proper polarity. Power was applied to the Mini- PROTEIN 3 cell and electrophoresis was done at 100 volts for approximately 90min until the bromophenol tracking dye reached the edge of the separating gel

(Baines, 2001)

5.2.4 Staining

The gel was carefully removed from the spacer plate and placed in a staining petri dish with 0.2% Coomassie stain (Appendix 2) and soaked overnight in a staining boat on an agitator shaker, Bioline, Australia). Then it was de-stained with several changes of de-staining solution (Appendix 2) until the background was clear (Baines, 2001). After the background of gel became clear it was placed in a zip-locked bag and images were captured with a scanner and digital camera.

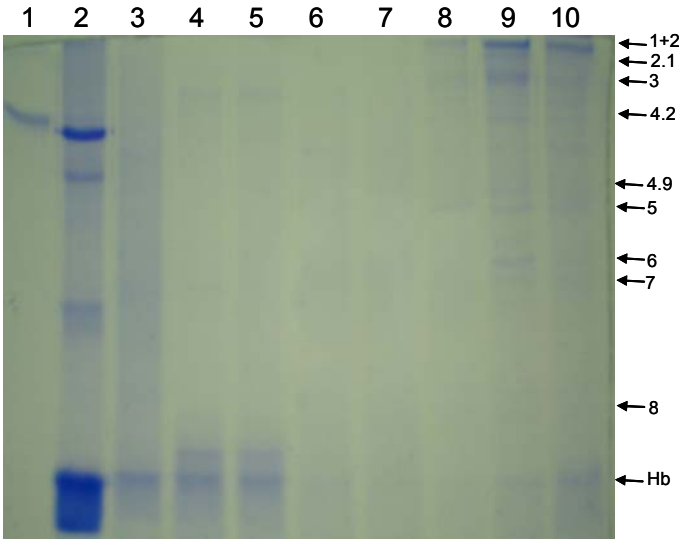
5.2.5 Results

The SDS- gel electrophoresis pattern of proteins separated from normal and MO affected red cell membranes with and without treatment of various toxins are presented in Figures 5.01 - 5.03. Although the quality of the gel representing extract activity on normal red cell membrane proteins is poor (Figure 5.01), there seems to be no discernable difference between that and MO affected red cells.

The extract isolated from the haemolytic yeast effected the red cell membranes the same, regardless of them being MO affected (row 2, Figure 5.01, row 3 Figure 5.02, row 3 Figure 5.03). Four high density bands are present at approximately 70,000 Da, 50,000 Da, 27,000 Da and 23,000 Da with some smearing of other bands, this may indicate cleavage of bands higher than 90,000Da, although it appears (Figure 5.02 and 5.03), that the large structural proteins (bands 1 - 2.1) are present.

Similarly, the rows that represent red cell membrane protein separation after incubation with toxin extract from *P. steckii* demonstrate abnormal band patterns compared to the controls. The extract isolated from *P. steckii* grown on rice (row 4 Figure 5.02 and row 4 Figure 5.03) has caused a smearing of bands suggesting non-specific protein destruction. The rows representing the effect of toxin isolated from *P. steckii* grown on wheat on red cell membranes demonstrates a more specific action (row 5 and 6, Figures 5.02 and Figures 5.03), particularly against the structural proteins of bands 1 - 2.1 (approximately 90,000 to 260,000 Da), this has resulted in a accumulation of smaller bands below 50,000 Da in size.

The effect of toxin extracts from *C. perfringens* on red cell membranes is demonstrated in rows 7 and 8 of Figures 5.02 and 5.03. It appears that the affect on MO case 1 cells is minimal (rows 7 and 8, Figure 5.02) as the band pattern is very similar to that of the normal, unaffected control. In comparison the rows that represent *C. perfringens* toxin incubated with MO case 2 red cell membranes (Row 7 and 8 Figure 5.03) seems to have cleaved bands 1 to 4.2 with resulting smearing indicating very small proteins as a result. This result seems incongruous and needs repeating. Regardless, the action of *C. perfringens* does not mimic exactly the action of *P. steckii*, therefore novel mechanisms may be involved.

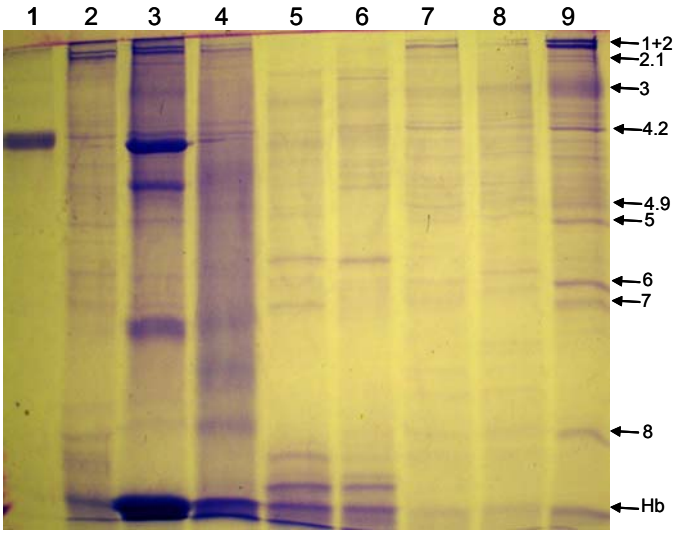


Band No.	Protein name	Molecular weight (Da)
1	α-spectrin	260,000
2	β-spectrin	225,000
2.1	Ankyrin	215,000
3	-	89,000
4.2	Palladin	72,000
4.9	Demantin	48,000
5	Actin	43,000
6	G3PD	35,000
7	-	29,000
8	-	23,000

Figure 5.01 SDS-PAGE profile of erythrocyte membrane treated with various toxins. Result shows different toxins have cleaved different protein fractions of RBC membrane.

Table 5.01 Details of samples used in different lanes.

Lane No.	Description
1	Bovine serum albumin
2	Normal control + yeast toxin extracted from BA
3	Normal control + *P. steckii* toxin extracted from rice
4	Normal control + *P. steckii* toxin extracted from wheat
5	Normal control + *P. steckii* toxin extracted from wheat
6	Normal control + *C. perfringens* toxin isolated from sago
7	Normal control + *C. perfringens* toxin extracted from ATCC 13124
8	MO case 2
9	MO case 1
10	Normal control

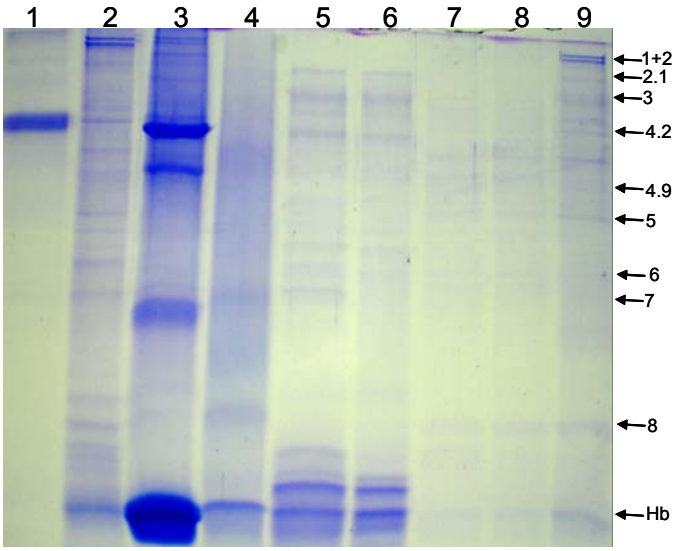


Band No.	Protein name	Molecular weight(Da)
1	α-spectrin	260,000
2	β-spectrin	225,000
2.1	Ankyrin	215,000
3	-	89,000
4.2	Palladin	72,000
4.9	Demantin	48,000
5	Actin	43,000
6	G3PD	35,000
7	-	29,000
8	-	23,000

Figure 5.02 SDS-PAGE profile of erythrocyte membrane treated with various toxins. Result shows different toxins have cleaved different protein fractions of RBC membrane.

Table 5.02 Details of samples used in different lanes.

Lane No.	Description
1	Bovine serum albumin
2	Normal control
3	MO case 1 + yeast toxin extracted from BA
4	MO case 1 + *P. steckii* toxin extracted from rice
5	MO case 1 + *P. steckii* toxin extracted from wheat
6	MO case 1 + *P. steckii* toxin extracted from wheat
7	MO case 1 + *C. perfringens* toxin isolated from sago
8	MO case 1 + *C. perfringens* toxin extracted from ATCC 13124
9	MO case 1



Band No.	Protein name	Molecular weight (Da)
1	α-spectrin	260,000
2	β-spectrin	225,000
2.1	Ankyrin	215,000
3	-	89,000
4.2	Palladin	72,000
4.9	Demantin	48,000
5	Actin	43,000
6	G3PD	35,000
7	-	29,000
8	-	23,000

Figure 5.03 SDS-PAGE profile of erythrocyte membrane treated with various toxins. Result shows different toxins have cleaved different protein fractions of RBC membrane.

Table 5.03 Details of samples used in different lanes.

Lane No.	Description
1	Bovine serum albumin
2	Normal control
3	MO case 2 + yeast toxin extracted from BA
4	MO case 2 + *P. steckii* toxin extracted from rice
5	MO case 2 + *P. steckii* toxin extracted from wheat
6	MO case 2 + *P. steckii* toxin extracted from wheat
7	MO case 2 + *C. perfringens* toxin isolated from sago
8	MO case 2 + *C. perfringens* toxin extracted from ATCC 13124
9	MO case 2

5.3 Discussion

From the observation of the SDS- gel electrophoretic protein patterns of the ghost cells treated with the different toxins, it was obvious that there was alteration in the bands patterns. However, the patterns are different from each other, indicating that different toxins had an affect on different proteins of the red cell membrane.

These data support the semi-quantifiable results of Chapter 4, which suggests that the organisms selected do indeed cause destruction of red cells, probably by attacking the red cell membranes. Given that this is the case, the active compounds in the extractions may be proteases or lipoproteases, similar compounds to other known haemolysins (Chapter 2) and this information may lead further investigation designed to characterise the compounds.

Although these data are suggestive of significant results, alterations in the experimental design would have helped in the interpretation. The extracts used to incubate with red cell membranes should have been run on a SDS PAGE gel without red cell ghosts so the size and complexity of the extract could be discerned. This would help determine if extra bands of the gels observed resulting from incubated extract and red cell membranes was toxin or the result of cleavage of red cell bands or toxin binding to bands. Also, although the SDS PAGE technique was undertaken using standard techniques, a silver stain as well as Coomassie blue would have aided in more clear band differentiation. To ensure that the intensity of bands could be compared, the protein concentration of each product to run on the SDS PAGE gel should have been standardised. These alterations would have enabled a more clear description of the results.

Not with standing the above problems, the extracts tested against red cell membranes did demonstrate activity consistent with the clinical findings of acute intravascular haemolysis, that is, aggressive structural membrane destruction. Therefore, the extracts contain compounds related to proteases or lipoproteases and therefore required further elucidation. This work supports the notion that *P. steckii* is the most likely of the aetiological agents to be implicated in clinical SHD. It seems the extract taken from *P. steckii* grown on rice has a more non-specific action as the resultant

smearing indicates non-specific activity against a number of bands resulting in very small fragments. The non-specificity may also reflect non-specific, artefactoral affects of reagents or contaminating compounds within the crude extract. Clearly, this work would benefit from access to a more purified compound and the scrutiny of a red cell protein chemist. The observation that the extract from *P. steckii* grown on wheat seemed to cleave the important structural proteins of the red cell membrane (α and β spectrin) result may also reflect the higher activity the extract had on red cells demonstrated in the semi-quantified assay in Chapter 4. Regardless, the activity of the extract from *P. steckii* incubated with wheat to cleave important structural proteins of α and β spectrin (Row 5 and 5 Figure 5.02 and 5.03) is interesting and deserves more thorough investigation.

Alpha - toxin, produced by *C. perfringens* is known to hydrolyze phosphatidylcholine present in the erythrocyte membrane (Mollyby, 1978; Sakurai *et al.*, 1993; Nagahama *et al.*, 1997). Several studies have demonstrated that biphasic production of 1,2- diacylglycerol and phosphatidic acid (PA) (Sakurai *et al.*, 1993; Ochi *et al.*, 1996) occured when rabbit erythrocyte was incubated with alpha toxin of *C. perfringens*. During that incubation, two PA were formed: the rapid PA formation and late PA formation. It was found that the rapid PA formation occurred due to the stimulation of endogenous PLC regulated by GTP [Guanosine 5'- *O*-(3-thiotriphosphate)] binding protein present in erythrocyte membrane (Sakurai *et al.*, 1993; Sakurai *et al.*, 1994). The late PA formation occurred on stimulation of the endogenous phospholipase D (PLD) (Sakurai *et al.*, 1994). It was also suggested that the possibility of the late PA formation could have occurred due to the activation of rapid PA formation. Therefore, it was postulated that GTP - binding protein may play a role in the late PA formation and this formation was found to be directly proportional to the haemolysis (Ochi *et al.*, 1996). The *C. perfringens* used in our experiments gave inconclusive results given it seems with MO case 2, significant structural protein was cleaved, this did not seem to be the case in MO case 1. As mentioned, the gel representing normal red cells was of poor quality and could not be confidently interpreted.

The unidentified yeast in this experiment, was isolated from sago, two weeks old, stored in a bag made of tree bark, from Kubut village, Middle Fly region, Western

Province, PNG. It was grown on BA and the extract presented a completely different protein pattern than the other toxin-ghost membrane complex after incubation. The spectrin (α and β) bands appeared normal but there was presence of a intensely stained, distinct and broad band of molecular weight of approx. 65 kDa. Similarly, another less intense and slimmer but clear band of approx. molecular weight of 55 kDa is present in the gel. Moreover, another faint but broad band of a approximate molecular weight of 30 kDa is present and finally a very wide band was found in the place of haemoglobin band.

Regarding the unique protein band pattern present in gel, this may be indication of blood contamination from BA medium. During the extracting procedure, whole BA medium was blended along with the yeasts. Possibly, this toxin might have acted on the blood from the medium. To avoid or minimize this contamination, pure colonies of yeast should be taken from the culture medium and alternative toxin extracting method is recommended.

The results suggest that there is no difference in haemolytic activity of toxins against normal and MO red cell membranes. This further suggests that MO is not a prediposing factor to SHD. Although, the toxin extracts used were crude and this non-specificity may have clouded the results. Clearly, the work needs to be repeated when the crude extracts are further fractionated and characterised before any final assumptions can be made.

CHAPTER 6 - GENERAL DISCUSSION

According to Bruce and co-workers, the prevalence of MO in coastal region of PNG is up to 40% (Bruce *et al.*, 2000). Melanesian ovalocytosis is a hereditary disorder of band 3 in RBCM (Tanner *et al.*, 1991; Sarabia *et al.*, 1993; Liu *et al.*, 1994; Bruce *et al.*, 2000). In PNG, sago haemolytic disease epidemic is frequently reported (Taufa, 1974; Donovan *et al.*, 1977). Sago is an important part of diet in this country. Therefore, observation of all these factors, the hypothesis of MO could be disposing factor for sago haemolytic disease can be postulated. Hence, if this work is able to establish this relationship, it could be utilized in prevention and control of this fatal disease.

In the first part of this study, it was detected that toxins extracted from organisms, which were isolated from sago, collected from PNG, possess haemolytic properties. However, the intensity of haemolysis is different in different toxins. The toxins were extracted using different methods according to their sources. Fungal and yeast toxin was extracted by utilizing method which is established by Rizzo and colleague (Rizzo *et al.*, 1992). On the other hand, toxins from bacteria were extracted utilizing the methods described by Reynaud and colleague (Jolivet-Reynaud *et al.*, 1988).

Fungal toxin is found to be more haemolytic than the clostridial and yeast toxin. Fungal toxin extracted from wheat has shown intense haemolysis than its counterpart from rice. *Clostridium perfringens* toxin has shown that it possesses haemolytic properties but is lesser than fungal toxin. A wide range of work has already elucidated the mechanism of haemolysis (Mollyby, 1978; Sakurai *et al.*, 1993; Nagahama *et al.*, 1997).

In comparison to the other two toxins, yeast toxin has presented little haemolytic activity. Many researchers have done a lot of study regarding this subject and they demonstrated the haemolytic property and mechanism of yeast toxin on RBC (Manns *et al.*, 1994; Soszynski and Schuessler, 1998; Watanabe *et al.*, 1999; Luo *et al.*, 2001).

The haemolytic assay in this work, has followed the method established by

Bernheimer (Bernheimer, 1988). The assay performed in this work appeared to be reliable because positive and negative control were also included. Positive control, mixture of PBS, 10% Triton X-100 and RBC suspension has given complete haemolysis. Negative control, mixture of PBS and RBC suspension has given no haemolysis or negligible haemolysis.

It was indicated from this result that the haemolytic activity of toxins varies according to the type of toxins, source, the culture medium, and extracting methods. This preliminary study was unable to analyze the detail of all toxins due to its time constraint and limited credit hours of study. However, it will be an interesting topic for further work.

Moreover, in the second part of this study, the SDS-gel electrophoretic pattern of the toxin- treated protein shows no apparent difference between alteration on the pattern of normal RBCM and MO RBCM. It is apparent that there is difference in the electrophoretic pattern of normal control and other two MO cases before toxin treatment. Difference between the electrophoretic pattern of MO 1 and MO 2 is also obvious, but due to the lack of other detail clinical history and reports, it is inappropriate to presume and comment anything regarding this issue. This requires more specific and detail study in this context.

Regardless of their different protein patterns before the toxin treatment, electrophoretic pattern of toxin treated sample presented similar pattern. This is prominent and unusual picture, which should be studied further. Again, this is only a preliminary study, therefore, unable to explain specific site and mechanism of toxin effect on protein fraction RBCM. However, the result of this study can be utilized further for comprehensive study and to elucidate the fact behind the sago haemolytic disease.

APPENDICES

APPENDIX 1

MEDIA AND REAGENTS OF HEMOLYSIN PRODUCTION

Culture medium for *Clostridium perfringens*

Protease Peptone (Oxoid, England)	25 g
Yeast extract (Oxoid, England)	6 g
Deionized water	150 ml

Mix the ingredients well and dialyse for 3 days in cold against 1 litre of deionized water.

Suppliments for culture media

Cysteine hydrochloride (Sigma)	2 g
$NaHCO_3$ (Sigma)	10 g
Dextrin (Sigma)	20 g
Acid- hydrolysed casein (casamino acids) (Difco)	10 g
KH_2PO_4 (BDH)	2 g

Add all the ingredients in dialysate and adjust pH to 6.7 with 3 N NaOH. Sterilized at 115˚C for 20 min in a container. The container is allowed to cool and kept in cold (at 4ºC) until inoculation.

TGY medium

Trypticase soya broth (Oxoid, England)	3 g
D-Glucose (BDH)	1 g
Yeast extract (Oxoid, England)	2 g
Deionized water	100 ml

Mix the ingredients and adjust the pH at 7.5.

Buffer A

Tris- HCl (BDH), 20 mM (pH 7.2)	2.428 g
NaCl (Ajax Finechem), 150mM	8.77 g
ZnCl2 (Sigma), 1mM	0.136 g
Deionized water	1000 ml

Ethanolic solution of cholesterol

Cholesterol acetate (Sigma)	20 mg
Ethanol 99.5% purity (Finechem)	1 ml

Reagents for toxin extraction

10 x Toxin Diluting Solution (TDS)

100 mM Tris (pH 7.2)
1.5 M NaCl (Ajax Finechem)
100 mM $MgCl_2$ (Sigma)
100 mM $CaCl_2$ (Sigma)

Toxin/Ethanol Solution, 100 ml

10 x Toxin diluting solution (TDS)	10 ml
Deionized water	20 ml
Ethanol	70 ml

10 x Phosphate buffer saline (PBS)

NaCl (Ajax Finechem)	80 g
KH_2PO_4 (BDH)	2 g
Na_2HPO_4 (BDH)	11.5 g
Distilled water upto	1000 ml

Combine all the ingredients and mix well and adjust the pH to 7.2

Sago Agar

Yeast Extract	7 g
Sago	50 g
$MgSO_4.7H_2O$	0.5 g
$CuSO_4$	0.005 g
$ZnSO_4$	0.001 g
Agar	15 g
dH_2O	1 lit
pH 6.5	

Use both Sigma and Difco yeast in separate batches of media. Sigma for sago S and Difco for sago D.

Potato Carrot Agar

Sliced potato	20 g
Sliced carrot	20 g
Agar	15 g
Tap water	1 lit
Chloramphenicol	100 mg

APPENDIX 2

REAGENTS AND BUFFERS FOR SDS-PAGE

10% sodium dodecyl sulphate (SDS)

Lauryl sulphate (Sigma)	10 g
Deionized water	100 ml

Dissolve 10g of SDS in 90 ml of deionized water with gentle mixing and make the final volume to 100ml.

Acrylamide Stock; 37.5:1(2.6%C) Toxic when un- polymerised.

Acrylamide/Bis (Bio-rad)	30 g
Deionized water	100 ml

Dissolve acrylamide and bisacrylamide in 70 ml of water. Make up volume to 100 ml, filter through a 0.45 µm filter. Store at 4ºC for up to 4 weeks in dark. Polymerised before disposal.

10% Ammonium Persulphate (APS) solution

Ammonium persulphate (Bio-Rad)	100 mg
Deionized water	1 ml

Dissolve in 1ml deionized water. Store at 4ºC and use within 24 hrs.

Separating Gel, 12% (10ml)

Deionized water	3.4 ml
30% acrylamide/bis (Bio-Rad)	4.0 ml
1.5M Tris-HCl buffer, (pH 8.8)	2.5 ml
10% SDS solution (w/v)	0.1 ml
10% APS solution (w/v)	50 µl
TEMED (Sigma)	5 µl

Combine all ingredients well, adding TEMED immediately prior to pouring the gel. Total volume of ingredients is sufficient to make one gel.

Stacking Gel, 4%(10ml)

Deionized water	6.1 ml
30% acrylamide/bis(Bio-Rad)	1.3 ml
0.5M Tris-HCl buffer, pH 6.8	2.5 ml
10% SDS solution (w/v)	0.1 ml
10% APS solution (w/v)	50 µl
TEMED (Sigma)	10 µl

Combine all ingredients well, adding TEMED immediately prior to pouring the gel. Total volume of ingredients is sufficient to make one gel.

1.5 M Tris-HCl (pH6.8)

Tris base (BDH)	18.15 g
Deionized water	100 ml

Adjust to pH 8.8 with 6N HCl. Make the volume up to 100ml with deionized water and store at 4ºC.

0.5 M Tris-HCl (pH 6.8)

Tris base (BDH)	6 g
Deionized water	100 ml

Adjust pH with 6N HCl. Make the volume up to 100 ml with deionized water and store at 4ºC.

10x Electrode (Running) Buffer (pH 8.3)

Tris Base (BDH)	30.3 g
Glycine (Sigma)	144 g
Lauryl sulphate (Sigma)	10 g
Deionized water	1000 ml

Mix ingredients well and store at 4ºC. Prior to use, dilute 50 ml of 10X stock with 450 ml deionized water.

SDS-PAGE Reducing Sample Buffer (2x)

0.5M Tris-HCl buffer (pH 6.8)	2.5 ml
10%SDS	4.0 ml
Glycerol (Ajax Finechem)	2.0 ml
Bromophenol blue (Progen)	2.0 mg
DL-Dithiothreitol (Promega)	0.31g
Deionized water	10 ml

Combine ingredients and mix well. Store in 0.5 ml aliquots at –20º C for up to 6 months

Coomassie Brilliant Blue Staining Solution

Coomassie Brilliant Blue R-250 (Sigma)	2 g
Methanol	500 ml
Acetic acid	100 ml
Deionized water	400 ml

Combine ingredients in fume hood. Store in brown bottle at room temperature away from direct light.

Coomassie Brilliant Blue De-staining Solution

Methanol 99.8% purity (APS, Finechem)	500 ml
Acetic acid (Finechem)	35 ml
Deionized water	415 ml

Combine ingredients in fume hood. Store at room temperature.

APPENDIX 3

HEMOLYTIC ASSAY TABLES

Table A3.01 Absorbance of hemolytic assay of rice and wheat extract with ethanol

Details	**Incubation time (min)**			
	30	**60**	**120**	**240**
Rice	106.1	102.1	100.6	94.9
	93.9	104.5	100.6	106.9
	98.8	115.9	115.9	106.6
Mean	**99.6**	**107.5**	**105.7**	**102.8**
SD	**6.14**	**7.37**	**8.83**	**6.84**
Wheat	95.2	79.6	84.9	85.5
	87.8	79.6	84.9	80
	85.8	75.8	87.1	73.9
Mean	**89.60**	**78.33**	**85.63**	**79.8**
SD	**1.90**	**1.93**	**1.12**	**3.47**

Table A3.02 Absorbance of hemolytic assay of *Penicillium steckii* from rice

Details	**Incubation time (min)**					
	30	**60**	**120**	**240**	**360**	**480**
P.steckii from rice with TDS	4.3	6.9	5.4	3.9	3.8	4.3
	6.8	7.1	7.2	6.6	4.6	5.1
	7.2	9	7.9	7.3	4.3	5.7
Mean	**6.1**	**7.67**	**6.83**	**5.93**	**4.23**	**5.03**
SD	**1.57**	**1.16**	**1.29**	**1.8**	**0.4**	**0.7**
P.steckii from rice with TDS	1.78	1.2	1.6	4.2	16.6	43.5
	2	1.8	1.6	5.4	17.2	48.9
	2	1.8	2.6	5.6	16.4	48.8
Mean	**1.93**	**1.6**	**1.93**	**5.07**	**16.73**	**47.7**
SD	**0.13**	**0.35**	**0.58**	**0.76**	**0.42**	**3.09**
P.steckii from rice with TDS/ethanol	10.8	7.9	10.4	11.4	11.6	13.4
	17.1	17.2	15.8	19	18.8	21.5
	12.3	14.7	14.8	15.9	15.9	18.1
Mean	**13.4**	**13.27**	**13.67**	**15.43**	**15.43**	**17.76**
SD	**3.29**	**4.81**	**2.87**	**3.82**	**3.62**	**4.07**

Table A3.03 Absorbance of hemolytic assay of *Penicillium steckii* from wheat

Details	**Incubation time (min)**					
	30	**60**	**120**	**240**	**360**	**480**
P.steckii from wheat with TDS	24.2	23.8	23.3	83.2	89	82.1
	22.1	22.4	22.4	75.3	88.1	74.9
	29.9	30.8	29	78.1	85.9	74.9
Mean	**25.4**	**25.67**	**24.90**	**78.87**	**87.67**	**77.30**
SD	**4.04**	**4.50**	**3.58**	**4.01**	**1.59**	**4.16**
P.steckii from wheat with TDS	59.5	83.9	99.9	72.4	54.8	48.2
	75.6	91.7	93.7	71.4	53.5	48.2
	86.4	94.2	93.7	66.3	48.7	43.5
Mean	**73.83**	**89.93**	**95.77**	**70.03**	**52.33**	**46.63**
SD	**13.54**	**5.37**	**3.58**	**3.27**	**3.21**	**2.71**
P.steckii from wheat with TDS	0.17	0.3	0.17	0	0.16	0.7
	0.2	0.1	0.4	0.05	0.3	0.6
	0.1	0.08	0.37	0.02	0	0.4
Mean	**0.16**	**0.16**	**0.31**	**0.02**	**0.15**	**0.57**
SD	**0.05**	**0.12**	**0.13**	**0.03**	**0.15**	**0.15**
P.steckii from wheat with TDS/Ethanol	12.8	14.6	14.7	14.8	14	15.5
	14.8	16.3	14.5	15.3	15.2	18.3
	15.5	18.8	18.9	18.6	17.9	32
Mean	**14.37**	**16.57**	**16.03**	**16.23**	**15.70**	**21.93**
SD	**1.40**	**2.11**	**2.48**	**2.06**	**2.00**	**8.83**

Table A3.04 Absorbance of hemolytic assay of *Candida spp* from sago D

Details	**Incubation time (min)**					
	30	**60**	**120**	**240**	**360**	**480**
Candida spp. from sago D with TDS	3	1.2	2.2	4.4	6.3	8.3
	3	3.5	2.9	3.4	5.8	5.3
	2.6	3.2	3.3	5.9	6.6	8.4
Mean	**2.87**	**2.63**	**2.80**	**4.57**	**6.23**	**7.33**
SD	**0.23**	**1.25**	**0.56**	**1.26**	**0.40**	**1.76**
Candida spp. from sago D with TDS/ Ethanol	15.3	15.5	16.8	15.1	18.2	18.4
	14.4	14.5	17.8	13.2	19.6	20.7
	16.7	17.7	20.1	14.7	22.5	27.8
Mean	**15.47**	**15.90**	**18.23**	**14.33**	**20.10**	**22.30**
SD	**1.16**	**1.64**	**1.69**	**1**	**2.19**	**4.9**

Table A3.05 Absorbance of hemolytic assay of *Candida spp* from sago S and blood agar

Details	**Incubation time (min)**					
	30	**60**	**120**	**240**	**360**	**480**
Candida spp. from sago S with TDS	3.2	2.6	2.6	5.2	7.1	6.8
	3.2	2.6	2.4	4.5	5.7	7.5
	3.3	3.1	2.2	3.9	5.7	8.3
Mean	**3.23**	**2.77**	**2.40**	**4.53**	**6.17**	**7.53**
SD	**0.06**	**0.29**	**0.20**	**0.65**	**0.81**	**0.75**
Candida spp. from BA with TDS	-0.5	5.7	31.3	98.1	85.6	84
	0.1	3.4	40.4	87.9	89.3	81.2
	1.5	8.3	51.7	90.8	94.2	84.6
Mean	**0.37**	**5.80**	**41.13**	**92.27**	**89.70**	**83.27**
SD	**1.03**	**2.45**	**10.22**	**5.26**	**4.31**	**1.81**
Candida spp. from BA with TDS/Ethanol	22.4	16.1	17.4	18.7	22.3	39.7
	20.5	19.1	16.4	20.4	22.9	37
	24	20.8	16.7	20.4	23.2	37.2
Mean	**22.30**	**18.67**	**16.83**	**19.83**	**22.80**	**37.97**
SD	**1.75**	**2.38**	**0.51**	**0.98**	**0.46**	**1.5**

REFERENCES

Council for Agricultural Science and Technology. (2003) Mycotoxins: Risks in Plant, Animal and Human Systems.

Argiolas, A. and Pisano, J.J. (1983) Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom. *J Biol Chem* **258**: 13697-702

Awad, M.M., Ellemor, D.M., Boyd, R.L., Emmins, J.J. and Rood, J.I. (2001) Synergistic effects of alpha-toxin and perfringolysin O in *Clostridium perfringens*-mediated gas gangrene. *Infect Immun* **69**: 7904-10

Baines, D. (2001) Protein Purification Techniques, 27-49 pp. second Edition ed. Analysis of purity, edited by S. Roe. Oxford University Press, UK.

Beecher, D.J. and Wong, A.C. (2000) Cooperative, synergistic and antagonistic haemolytic interactions between haemolysin BL, phosphatidylcholine phospholipase C and sphingomyelinase from Bacillus cereus. *Microbiology* **146**: 3033-9.

Berkowitz, F.E. (1991) Hemolysis and Infection: Categories and Mechanisms of Their Interrelationship. *Reviews of Infectious Diseases* **13**: 1151-62

Bernheimer, A.W. (1988) Assay of hemolytic toxins. *Methods Enzymol* **165**: 213-7.

Betina, V. (1984a) Mycotoxins, Production, Isolation, Separation and Purification, 217-236 pp. Citrinin and related substances, edited by V. Betina, 8. 8 vols. Elsevier Science Publishers B.V., Armsterdam, Netherlands.

Betina, V. (1984b) Mycotoxins, Production, Isolation, Separation and Puritfication, 3-12 pp. Toxigenix Fungi and their occurence, edited by V. Betina, 8. 8 vols. Elsevier Science Publishers B.V, Amsterdam, Netherlands.

Bhakdi, S. and Tranum-Jensen, J. (1985) Complement activation and attack on autologous cell membranes induced by streptolysin-O. *Infect Immun* **48**: 713-9.

Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985) Mechanism of membrane damage by streptolysin-O. *Infect Immun* **47**: 52-60

Blackman, S.M., Hustedt, E.J., Cobb, C.E. and Beth, A.H. (2001) Flexibility of the cytoplasmic domain of the anion exchange protein, band 3, in human erythrocytes. *Biophys J* **81**: 3363-76.

Bruce, L.J., Wrong, O., Toye, A.M., Young, M.T., Ogle, G., Ismail, Z., Sinha, A.K.,

McMaster, P., Hwaihwanje, I., Nash, G.B., Hart, S., Lavu, E., Palmer, R., Othman, A., Unwin, R.J. and Tanner, M.J. (2000) Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malaysia and Papua New Guinea: loss of up to 95% band 3 transport in red cells. *Biochem J* **350**: 41-51.

Bryant, A.E. and Stevens, D.L. (1996) Phospholipase C and perfringolysin O from *Clostridium perfringens* upregulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce interleukin-8 synthesis in cultured human umbilical vein endothelial cells. *Infect Immun* **64**: 358-62

Chang, S.H. and Low, P.S. (2001) Regulation of the glycophorin C-protein 4.1 membrane-to-skeleton bridge and evaluation of its contribution to erythrocyte membrane stability. *J Biol Chem* **276**: 22223-30.

Chart, H., Jenkins, C., Smith, H.R., Hedges, D. and Rowe, B. (1998) Haemolysin production by strains of Verocytotoxin-producing *Escherichia coli*. *Microbiology* **144**: 103-7.

Cho, M.R., Knowles, D.W., Smith, B.L., Moulds, J.J., Agre, P., Mohandas, N. and Golan, D.E. (1999) Membrane dynamics of the water transport protein aquaporin-1 in intact human red cells. *Biophys J* **76**: 1136-44.

Davidson, F.F. and Dennis, E.A. (1991) Reptile Venoms and Toxins, 107 - 145 pp. Structure, Function, and Mode of Action of Snake Venom and Other Phospholipases A2, edited by A.T. Tu, 5. 6 vols. Marcell Dekker, Inc., New York.

Davis, N.D., Wagener, E., Dalby, D.K., Morgan-Jones, G. and Diener, U.L. (1975) Toxigenic fungi in food. *Appl Microbiol* **30**: 159-61.

Dennis, E., Darke, P., Deems, R., Kensil, C. and A., P. (1981) Cobra venom phospholipase A2: A review of its action toward lipid/water interfaces. *Molecular and Cellular Biochemistry*: 37 - 45

Deregnaucourt, C. and Schrevel, J. (2000) Bee venom phospholipase A_2 induces stage-specific growth arrest of the intraerythrocytic *Plasmodium falciparum* via modifications of human serum components. *J Biol Chem* **275**: 39973-80

Donovan, K., Shaw, D.E. and Amato, D. (1977) Sago haemolysis: clinical features and microbiological studies. *P N G Med J* **20**: 167-74

Ellis, B.A., Rotz, L.D., Leake, J.A., Samalvides, F., Bernable, J., Ventura, G.,

Padilla, C., Villaseca, P., Beati, L., Regnery, R., Childs, J.E., Olson, J.G. and Carrillo, C.P. (1999) An outbreak of acute bartonellosis (Oroya fever) in the Urubamba region of Peru, 1998. *Am J Trop Med Hyg* **61**: 344-9.

Fallon, K., Bausch, K., Noonan, J., Huguenel, E. and Tamburini, P. (1997) Role of aspartic proteases in disseminated Candida albicans infection in mice. *Infect Immun* **65**: 551-6.

Figueiredo, P.M., Catani, C.F. and Yano, T. (2003) Thiol-independent activity of a cholesterol-binding enterohemolysin produced by enteropathogenic *Escherichia coli*. *Braz J Med Biol Res* **36**: 1495-9

Garratty, G., Telen, M.J. and Petz, D.L. (2002) Red Cell Antigens as Functional Molecules and Obstacles to Transfusion. *American Society of Haematology*: 445 - 462

Gomez, A., Mve-Obiang, A., Vray, B., Rudnicka, W., Shamputa, I.C., Portaels, F., Meyers, W.M., Fonteyne, P.A. and Realini, L. (2001) Detection of phospholipase C in nontuberculous mycobacteria and its possible role in hemolytic activity. *J Clin Microbiol* **39**: 1396-401

Grant, K.A., Belandia, I.U., Dekker, N., Richardson, P.T. and Park, S.F. (1997) Molecular characterization of pldA, the structural gene for a phospholipase A from *Campylobacter coli*, and its contribution to cell-associated hemolysis. *Infect Immun* **65**: 1172-80

Gray, L.D., Mackman, N., Baker, K., Nicaud, J.-M. and Holland, I.B. (1988) Bacterial Protein Toxins, 159 - 160 pp. The Secretion of *E. Coli* Haemolysin, edited by F.J. Fehernbach, J.E. Alouf, P. Falmagne, W. Goebel, J. Jeljaszewicz, D. Jurgens and R. Rappuoli. Gustav Fischer, Stuttgart, New York.

Habermehl, G.G. (1981) Venomous Animals and Their Toxins, 130 - 179 pp. Reptilia (Reptiles). Berlin Heidelberg, Germany.

Heinrich, V., Ritchie, K., Mohandas, N. and Evans, E. (2001) Elastic thickness compressibilty of the red cell membrane. *Biophys J* **81**: 1452-63.

Herwaldt, B.L., McGovern, P.C., Gerwel, M.P., Easton, R.M. and MacGregor, R.R. (2003) Endemic babesiosis in another eastern state: New Jersey. *Emerg Infect Dis* **9**: 184-8.

Hou, C.T., Ciegler, A. and Hesseltine, C.W. (1971) Tremorgenic toxins from penicillia. 3. Tremortin production by Penicillium species on various

agricultural commodities. *Appl Microbiol* **21**: 1101-3.

Jeljaszewicz, J., Szmigielski, S. and Hryniewicz, W. (1978a) Bacterial Toxins and Cell Membranes, 185 - 227 pp. Biological Effects of Staphylococcal and Streptococcal Toxins, edited by J. Jeljaszewicz and T. Wadstrom. Academic Press Inc., London.

Jeljaszewicz, J., Szmigielski, S. and Hryniewicz, W. (1978b) Biological Effects of Staphylococcal and Streptococcal Toxins, 185- 219 pp. Bacterial Toxins and Cell Membranes, edited by J. Jeljaszewicz and T. Wadstrom. Academic Press Inc., London.

Jolivet-Reynaud, C., Moreau, H. and Alouf, J.E. (1988) Purification of alpha toxin from Clostridium perfringens: phospholipase C. *Methods Enzymol* **165**: 91-4.

Khanna, R., Chang, S.H., Andrabi, S., Azam, M., Kim, A., Rivera, A., Brugnara, C., Low, P.S., Liu, S.C. and Chishti, A.H. (2002) Headpiece domain of dematin is required for the stability of the erythrocyte membrane. *Proc Natl Acad Sci U S A* **99**: 6637-42.

Kimura, M., Tamam, M., Soemantri, A., Nakazawa, M., Ataka, Y., Ohtsuka, R. and Ishida, T. (2003) Distribution of a 27-bp deletion in the band 3 gene in South Pacific islanders. *J Hum Genet* **48**: 642-5. Epub 2003 Nov 15.

Kingma, R.L. and Egmond, M.R. (2002) Activation of a covalent outer membrane phospholipase A dimer. *Eur J Biochem* **269**: 2178-85

Lee, S.H., Kim, S., Park, S.C. and J., K.M. (2002) Cytotoxic Activities of *Leptospira interrogans* Hemolysin SphH as a Pore-Forming Protein on Mammalian Cells. *Infect Immun* **70**: 315 - 322

Lenormand, G., Henon, S., Richert, A., Simeon, J. and Gallet, F. (2001) Direct measurement of the area expansion and shear moduli of the human red blood cell membrane skeleton. *Biophys J* **81**: 43-56.

Lim, H.W.G., Wortis, M. and Mukhopadhyay, R. (2002) Stomatocyte-discocyte-echinocyte sequence of the human red blood cell: evidence for the bilayer-couple hypothesis from membrane mechanics. *Proc Natl Acad Sci U S A* **99**: 16766-9.

Liu, S.C., Jarolim, P., Rubin, H.L., Palek, J., Amato, D., Hassan, K., Zaik, M. and Sapak, P. (1994) The homozygous state for the band 3 protein mutation in Southeast Asian Ovalocytosis may be lethal. *Blood* **84**: 3590-1.

Luo, G., Samaranayake, L.P. and Yau, J.Y. (2001) Candida species exhibit

differential in vitro hemolytic activities. *J Clin Microbiol* **39**: 2971-4

Maco, V., Maguina, C., Tirado, A. and Vidal, J.E. (2004) Carrion's disease (Bartonellosis bacilliformis) confirmed by histopathology in the High Forest of Peru. *Rev Inst Med Trop Sao Paulo* **46**: 171-4. Epub 2004 Jul 20.

Maguire, G.F., Lee, M. and Connelly, P.W. (1989) Sodium dodecyl sulfate-glycerol polyacrylamide slab gel electrophoresis for the resolution of apolipoproteins. *J Lipid Res* **30**: 757-61.

Malmstrom, J., Christophersen, C. and Frisvad, J.C. (2000) Secondary metabolites characteristic of Penicillium citrinum, Penicillium steckii and related species. *Phytochemistry* **54**: 301-9.

Manno, S., Takakuwa, Y. and Mohandas, N. (2002) Identification of a functional role for lipid asymmetry in biological membranes: Phosphatidylserine-skeletal protein interactions modulate membrane stability. *Proc Natl Acad Sci U S A* **99**: 1943-8.

Manns, J.M., Mosser, D.M. and Buckley, H.R. (1994) Production of a hemolytic factor by Candida albicans. *Infect Immun* **62**: 5154-6.

Maslow, J.N., Dawson, D., Carlin, E.A. and Holland, S.M. (1999) Hemolysin as a virulence factor for systemic infection with isolates of *Mycobacterium avium* complex. *J Clin Microbiol* **37**: 445-6

McKenzie, S.B. (1996) Textbook of Hematology, 213-274 pp. Second edition ed. Williams & Wilkins.

Meloni, T., Forteleoni, G., Dore, A. and Cutillo, S. (1983) Favism and Hemolytic Anemia in Glucose-6- Phoshate Dehydrogenase- Deficient Subjects in North Sardinia. *Clinica Pediatrica*

Melrose, W.D. (1990) Alcohol-associated hemolysis in Zieve's syndrome: A clinical and laboratory study of five cases. *Clin. Lab. Haemat.* **12**: 159 -167

Mollyby, R. (1978) Bacterial Phospholipases. In: J. Jeljaszewicz and T. Wadstrom (Eds), Bacterial Toxins and Cell Membranes, pp. 367- 415. Academic Press, London.

Moreau, C. (1979) Moulds, Toxins and Food, 3 -26 pp. Second ed. Food-borne moulds. John Willey & Sons Ltd., Paris.

Nagahama, M., Nakayama, T., Michiue, K. and Sakurai, J. (1997) Site-specific mutagenesis of *Clostridium perfringens* alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. *Infect*

Immun **65**: 3489-92

Neil, R.J. and Holmes, R.K. (1988) Bacterial Toxins, 383 - 416 pp. Genetics of Toxinogenesis in Bacteria, edited by M.C. Hardegree and A.T. Tu, 4. 5 vols. Marcel Dekker, New Yorl.

Ochi, S., Hashimoto, K., Nagahama, M. and Sakurai, J. (1996) Phospholipid metabolism induced by Clostridium perfringens alpha-toxin elicits a hot-cold type of hemolysis in rabbit erythrocytes. *Infect. Immun.* **64**: 3930-3933

Oliver, M., Coton, T., Badens, C., Dehan, C., Lena-Russo, D. and Moalic, J.L. (2001) Homozygous G6PD deficiency and propacetamol induced hemolysis. *Haematologica* **86**: 987-8

Picart, C., Dalhaimer, P. and Discher, D.E. (2000) Actin protofilament orientation in deformation of the erythrocyte membrane skeleton. *Biophys J* **79**: 2987-3000.

Quiros, S., Gene, J.A., Gutierrez, J.M. and Thelestam, M. (1992) Effect of Bothrops asper (fer- de- lance) snake venom on erythrocyte membrane: A comparative study. *Comp. Biochem. Physiol.* **101C**: 433 - 436

Rizzo, A.F., Atroshi, F., Hirvi, T. and Saloniemi, H. (1992) The hemolytic activity of deoxynivalenol and T-2 toxin. *Nat Toxins* **1**: 106-10

Rodak, B.F. (2002) Hematology Clinical Principles and applications. second ed. W.B. Saunders company.

Sakurai, J., Ochi, S. and Tanaka, H. (1993) Evidence for coupling of *Clostridium perfringens* alpha-toxin-induced hemolysis to stimulated phosphatidic acid formation in rabbit erythrocytes. *Infect Immun* **61**: 3711-8.

Sakurai, J., Ochi, S. and Tanaka, H. (1994) Regulation of *Clostridium perfringens* alpha-toxin-activated phospholipase C in rabbit erythrocyte membranes. *Infect Immun* **62**: 717-21.

Sarabia, V.E., Casey, J.R. and Reithmeier, R.A. (1993) Molecular characterization of the band 3 protein from Southeast Asian ovalocytes. *J Biol Chem* **268**: 10676-80.

Shahrokh, Z., Verkman, A.S. and Shohet, S.B. (1991) Distance between Skeleton Protein 4.1 and the Erythrocyte Membrane Bilayer Measured by Resonance Energy Transfer. *The Journal of Biological Chemistry* **266**: 12082- 12089

Soszynski, M. and Schuessler, H. (1998) Effect of ethanol and formate radicals on erythrocyte membrane proteins. *Int J Radiat Biol* **73**: 211-8.

Takasaki, C., Yanagita, A., Sugama, A. and Tamiya, N. (1987) Progress in Venom

and Toxin Research, 251 - 260 pp. First ed. Chemical Modifications of Phospholipase A_2 from the venom of King Brown Snake, Pseudechis Australis, edited by P. Gopalakrishnakone and C.K. Tan. Faculty of Medicine, National University of Singapore, Singapore.

Takeuchi, M., Miyamoto, H., Sako, Y., Komizu, H. and Kusumi, A. (1998) Structure of the erythrocyte membrane skeleton as observed by atomic force microscopy. *Biophys J* **74**: 2171-83.

Tanaka, W.T.H., Nakao, N., Mikami, T. and Matsumoto, T. (1997) Hemoglobin Is Utilized byCandida albicansin the Hyphal Form but Not Yeast Form. *Biochemical and Biophysical Research Communications* **232**: 350-353

Tanner, M.J., Bruce, L., Martin, P.G., Rearden, D.M. and Jones, G.L. (1991) Melanesian hereditary ovalocytes have a deletion in red cell band 3. *Blood* **78**: 2785-6.

Taufa, T. (1974) Sago Haemolytic Disease. *Papua New Guinea Medical Journal* **17**: 227- 228

Ubarretxena-Belandia, I., Cox, R.C., Dijkman, R., Egmond, M.R., Verheij, H.M. and Dekker, N. (1999) Half-of-the-sites reactivity of outer-membrane phospholipase A against an active-site-directed inhibitor. *Eur J Biochem* **260**: 794-800

Vesper, S.J., Dearborn, D.G., Yike, I., Sorenson, W.G. and Haugland, R.A. (1999) Hemolysis, toxicity, and randomly amplified polymorphic DNA analysis of Stachybotrys chartarum strains. *Appl Environ Microbiol* **65**: 3175-81

Virtanen, J.A., Cheng, K.H. and Somerharju, P. (1998) Phospholipid composition of the mammalian red cell membrane can be rationalized by a superlattice model. *Proc Natl Acad Sci U S A* **95**: 4964-9.

Vogel, C.W., Pluckthun, A., Muller-Eberhard, H.J. and Dennis, E.A. (1981) Hemolytic assay for venom phospholipase A2. *Anal Biochem* **118**: 262-8.

Waitumbi, J.N., Opollo, M.O., Muga, R.O., Misore, A.O. and Stoute, J.A. (2000) Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. *Blood* **95**: 1481-6

Wannamaker, L.W. and Schlievert, P.M. (1988) Bacterial Toxins, 267 - 295 pp. Exotoxins of Group A Streptococci, edited by M.C. Hardegree and A.T. Tu, 4. 5 vols. Marcel Dekker, Inc., New York.

Watanabe, T., Takano, M., Murakami, M., Tanaka, H., Matsuhisa, A., Nakao, N.,

Mikami, T., Suzuki, M. and Matsumoto, T. (1999) Characterization of a haemolytic factor from *Candida albicans*. *Microbiology* **145 (Pt 3)**: 689-94

Weatherall, D.J., Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K., Casals-Pascual, C. and Roberts, D.J. (2002) Malaria and the red cell. *Hematology (Am Soc Hematol Educ Program)*: 35-57

Wells, J.M. and Payne, J.A. (1975) Toxigenic Aspergillus and Penicillium isolates from weevil-damaged chestnuts. *Appl Microbiol* **30**: 536-40.

Woodrow, C.J., Burchmore, R.J. and Krishna, S. (2000) Hexose permeation pathways in *Plasmodium falciparum*-infected erythrocytes. *Proc Natl Acad Sci U S A* **97**: 9931-6.

Worlitzsch, D., Kaygin, H., Steinhuber, A., Dalhoff, A., Botzenhart, K. and Doring, G. (2001) Effects of amoxicillin, gentamicin, and moxifloxacin on the hemolytic activity of *Staphylococcus aureus* in vitro and in vivo. *Antimicrob Agents Chemother* **45**: 196-202

Yamamoto, I., Kimoto, H., Taketo, Y. and Taketo, A. (2001) Mutational and comparative analysis of streptolysin O, an oxygen-labile streptococcal hemolysin. *Biosci Biotechnol Biochem* **65**: 2682-9

Yawata, A., Kanzaki, A., Gilsanz, F., Delaunay, J. and Yawata, Y. (1997) A markedly disrupted skeletal network with abnormally distributed intramembrane particles in complete protein 4.1-deficient red blood cells (allele 4.1 Madrid): implications regarding a critical role of protein 4.1 in maintenance of the integrity of the red blood cell membrane. *Blood* **90**: 2471-81.

Zhang, D., Kiyatkin, A., Bolin, J.T. and Low, P.S. (2000) Crystallographic structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3. *Blood* **96**: 2925-33.