The Comparative Study of Sensitivity and Specificity of Techniques Used for Platelet Alloantibodies Detection

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biomedical Sciences

Prince of Songkla University

2009

Thesis Title	The Comparative Study of Sensitivity and Specificity of Techniques Used for Platelet Alloantibodies Detection
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ชื่อวิทยานิพนธ์	การศึกษาเปรียบเทียบความไวและความจำเพาะของการทดสอบในการตรวจหาแอนติบอดีที่จำเพาะต่อเกล็ดเลือด
ผู้เขียน	นายจรินทร์ บัวแก้ว
สาขาวิชา	ชีวเวชศาสตร์
ปีการศึกษา	2552

บทคัดย่อ

บทนำ ความผิดปกติที่เกิดจาก platelet - reactive antibodies มีความจำเป็นต้องวินิจฉัยและรักษาอย่างเหมาะสม แอนติบอดีที่เกี่ยวข้องส่วนใหญ่เป็น HLA class I antibody ส่วนน้อยเป็นแอนติบอดีต่อ human platelet antigen (HPA) ภาวะที่เกี่ยวข้องกับ platelet alloantibodies ได้แก่ platelet refractoriness, neonatal alloimmune thrombocytopenia (NAIT) และ post-transfusion purpura เทคนิคที่ใช้ในการตรวจหาแอนติบอดีควรทำได้รวดเร็ว มีความไวและความจำเพาะสูง ปัจจุบัน ในประเทศไทยนิยมใช้เทคนิค lympocytotoxicity test (LCT) ในการตรวจกรองแอนติบอดีต่อ HLA และตรวจความเข้ากันได้เพื่อปลูกถ่ายอวัยวะและการเลือกให้เกล็ดเลือดที่จำเพาะต่อผู้ป่วย และใช้เทคนิค solid phase red cell adherence assay (SPRCA) เพื่อใช้ในการตรวจวินิจฉัยการมีแอนติบอดีต่อเกล็ดเลือดทั้งชนิด HLA และHPA และการเลือกให้เกล็ดเลือดที่จำเพาะต่อผู้ป่วย วัตถุประสงค์ เพื่อเปรียบเทียบความไวและความจำเพาะของเทคนิคที่ใช้ในการทดสอบ platelet alloantibody โดยวิธี lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) และ flow cytometry และประเมินเปรียบเทียบความเหมาะสมของเทคนิคที่จะนำมาใช้ในงานบริการประจำวัน

วัสดุและวิธีการ ศึกษาจากตัวอย่างซีรัมจากผู้ป่วย 43 รายและจากกลุ่มควบคุม 10 ราย ซีรัมทุกรายได้รับการตรวจกับทั้ง 3 เทคนิคเพื่อหา HLA และ HPA antibodies โดยทำปฏิกิริยากับ screening lymphocyteหรือ platelet panel จำนวน 6 ราย จากนั้นนำผลการทดสอบมาวิเคราะห์หาความไว ความจำเพาะและความสอดคล้องกันของทั้ง 3 เทคนิค รวมทั้งคำนวณค่าใช้จ่ายและเวลาที่ใช้ในแต่ละเทคนิค

ผลการศึกษา จากการศึกษาตัวอย่างเลือด 43 รายตรวจโดยใช้ 3 เทคนิค พบ HLA antibody จำนวน 18 ราย เมื่อตรวจด้วย LCT พบจำนวน 17 รายเมื่อตรวจด้วย SPRCA ซึ่งเป็นผลบวกลวง 1 ราย และพบจำนวน 19 รายเมื่อตรวจด้วย flow cytometry ในการตรวจ HPA antibody เทคนิค flow cytometry ตรวจพบ 4 รายซึ่งมีเพียง 2 รายที่ตรวจพบได้โดย SPRCA เมื่อวิเคราะห์ความไวและ

ความจำเพาะในการตรวจหา HLA antibody พบว่า flow cytometry มีความไวและความจำเพาะสูงที่สุดเมื่อนำเทคนิค SPRCA และ LCT มาเปรียบเทียบ โดยพบความไว/ความจำเพาะของ SPRCA และ LCT เป็น 84.21%/95.83% และ 94.73%/100% ตามลำดับ และมีความสอดคล้องกันในระดับดี (K=0.76 และ K=0.81 ตามลำดับ) ส่วนความไวและความจำเพาะในการตรวจหา HPA antibody เมื่อเทียบกับ flow cytometry พบว่า SPRCA มีความไว 50% ความจำเพาะ 100% และในการตรวจ 1 ตัวอย่างด้วยเทคนิค LCT, SPRCA และ flow cytometry มีค่าใช้จ่ายเป็น 746, 735 และ 1,661 บาท ตามลำดับ และเวลาที่ใช้เป็น 8.0, 6.0 และ 4.0 ชั่วโมง ตามลำดับ

วิจารณ์ จากการศึกษาพบว่าเมื่อเปรียบเทียบกับเทคนิค SPRCA และ LCT เทคนิค flow cytometry มีความไวและความจำเพาะสูงสุดทั้งในการตรวจหา HLA antibody และ HPA antibody เทคนิค SPRCA ไม่สามารถตรวจพบ HPA antibodyในซีรัม 2 รายซึ่งเป็น idiopathic thrombocytopenic purpura (ITP) และมารดาของทารกที่อาจเป็น NAIT ในขณะที่เทคนิค flow cytometry สามารถตรวจพบได้ จะเห็นว่า SPRCA มีความไวไม่สูงพอต่อการวินิจฉัย NAIT ซึ่งมีผลกระทบต่อทารกสูงมาก เพราะเป็นโรคที่มีความรุนแรงจึงไม่ควรนำมาใช้ตรวจภาวะ NAIT ในการศึกษานี้เทคนิค SPRCA มีความไวต่ำมากซึ่งอาจเป็นจากมีจำนวนตัวอย่างศึกษาน้อยเกินไปทำให้ขาดความแม่นยำ แม้เทคนิค SPRCA และ LCT มีความไวต่ำกว่าเทคนิค flow cytometry แต่สามารถใช้ในการตรวจ platelet crossmatching ได้เพราะไม่ต้องการความแม่นยำสูงในการเลือกเกล็ดเลือดแก่ผู้ป่วย ไม่ต้องใช้เครื่องมือราคาแพง ส่วนในการตรวจ HLA antibody และ HLA crossmatchingในผู้ป่วยปลูกถ่ายอวัยวะ การใช้เทคนิคที่มีความไวต่ำอาจเกิด graft rejection ได้โดยเฉพาะในประเทศไทยยังตรวจด้วยเทคนิคLCT

สรุป flow cytometry เป็นเทคนิคที่มีความไวและมีความจำเพาะสูง และมีความเหมาะสมที่จะนำมาประยุกต์ใช้ในการตรวจหา platelet antibodiesในงานบริการประจำวัน

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Academic Year	2009

Abstract

Background: Platelet - reactive antibodies cause several disorders required appropriate diagnosis and specific treatments. Problematic antibodies are antibodies against HLA lass I and to the lesser extent human specific platelet antibody. Platelet refractoriness, neonatal alloimmune thrombocytopenia (NAIT) and post-transfusion purpura (PTP) are examples of disorders associate with platelet alloantibodies. Rapid diagnostic laboratory approach with sensitive and specific assays is necessary. In Thailand lymphocytotoxicity test (LCT) is commonly used for HLA antibody screening and crossmatching for both purposes, platelet antibody screen and organ transplantation. Solid phase red cell adherence assay (SPRCA) was used for platelet antibody screen and selection of platelet transfusion.

Objectives: To compare sensitivity and specificity of three methods tested for platelet reactive antibodies which were LCT, SPRCA and flow cytometry. Cost-effectiveness and performing time of these tests were also assessed to find out the suitable method for routine investigation.

Methods: Sera from 10 healthy controls and 43 samples from patients were screened for platelet reactive antibodies. All sera were tested in parallel by these 3 methods against the same six random lymphocyte or platelet screening panels. Sensitivity and specificity were assessed and level of agreement between pair of technique were also analysed. Cost and time consumption per test for each technique was analysed.

Results: The results showed that HLA reactive antibodies were found in 18 out of 43 sera using LCT assay, 17 out of 43 sera when using SPRCA with 1 sample was false reactive and 19 out of 43 sera when using flow cytometry. There were 4 out of 43 patients showed HPA reactive antibodies tested by flow cytometry technique but reactive only in 2 patients when using SPRCA

technique. The sensitivity and specificity of HLA antibody screening by three methods were analysed and found that flow cytometry had highest sensitivity and specificity. Compare to flow cytometry sensitivities/specificities of SPRCA and LCT were 84.21/95.83 % and 94.73/100% respectively with good strength of agreement (K= 0.76 and K= 0.81 respectively). Flow cytometry was also more sensitive than SPRCA in HPA antibody detection with 50% sensitivity and 100% specificity. The cost of LCT, SPRCA and flow cytometry technique was 746, 735 and 1,661 Baht, and time consumption was 8, 6 and 4 hours, respectively.

Discussion: In this study flow cytometry was a highest sensitive technique compare with SPRCA and LCT either screen for HPA antibody or HLA antibody. There were two patients whom provisional diagnoses were NAIT and idiopathic thrombocytopenia that SPRCA could not detect HPA antibodies while sera were reactive with flow cytometry. This is crucial that SPRCA was not sensitive enough to diagnose NAIT of which the risk of fetus/newborn is too high. According to our study SPRCA should not be used for NAIT testing. Although SPRCA sensitivity in our study was too low this is because of too few number of samples. SPRCA may still have a role in some places where flow cytometry test is not available and may be used for platelet crossmatching as it does not need expensive instrument. In our study we found that LCT test and SPRCA had lower sensitivity compare to flow cytometry. These detections did not have crucial effect for platelet crossmatching but these may have some effects on HLA antibody screening and crossmatching in potential organ transplant recipients and LCT is commonly used in Thailand.

Conclusion: The Flow cytometry technique appears to be sensitive and effective method of detecting platelet-reactive antibodies and well-suited for routine service.

ACKNOWLEDGEMENT

First of all, I would like to express my sincere thanks to my major advisor Dr. Charupon Promwong, Blood Bank and Transfusion Medicine Unit, Department of Pathology, Faculty of Medicine , Prince of Songkla University for her kindly and valuable advice and encouragement throughout the work in this thesis.

I also sincerely thank Miss Pawinee Kuptawintu and staff, HLA laboratory, National Blood Center, Thai Red Cross Society for some samples and technical support.

Special thanks are given to all members of Blood Bank Laboratory, Department of Pathology, Faculty of Medicine, Prince of Songkla University for their co-operation and assistance for laboratory work. Finally, I would like to express my deepest gratitude to my parents and thanks my friends for their love and support.

Jarin Buakaew

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LIST OF ABBREVIATION AND SYMBOLS

ACD	=	Acid citrate dextrose
ADP	=	Adenosine-5'- diphosphate
A LL	=	Acute lymphoblastic leukemia
AML	=	Acute myeloid leukemia
ATP	=	Adenosine-5'- triphosphate
BSA	=	Bovine serum albumin
CD	=	Cluster of differentiation
CML	=	Chronic myeloid leukemia
^{o}C	=	Degree celsius
DW	=	Distilled water
EDTA	=	Ethylenediaminetatraacitic acid
et al.	=	Et ali (Latin) and others
fl	=	Femtoliter
FITC	=	Fluorescein isothiocyanate
GTP	=	Guanosine -5'-triphosphate
HLA	=	Human leukocyte antigen
HPA	=	Human platelet antigen
IgG	=	Immunoglobulin G
IU	=	International unit
ITP	=	Idiopathic thrombocytopenic purpura
LCT	=	Microlymphocytotoxicity test
NAIT	=	Neonatal alloimmune thrombocytopenia
NSS	=	Normal saline solution
mm	=	Millimeter
M	=	Molar

LIST OF ABBREVIATION AND SYMBOLS (continue)

MHC	=	Major histocompatibility complex
ml	=	Milliliter
MDS	=	Myelodysplastic syndrome
p	=	p value
PBMC	=	Peripheral blood mononuclear cell
PBS	=	Phosphate buffer saline
PE	=	Phycoerythrin
PAF	=	Platelet-activating factor
PTP	=	Post transfusion purpura
R/O	=	Rule out
RPM	=	Round per minute
SPRCA	=	Solid phase red cell adherence assay
TNF	=	Tumor necrosis factor
µl	=	Microliter
µm	=	Micrometer
U	=	Unit
α	=	Alpha
β	=	Beta
γ	=	Gamma
κ	=	Kappa

CHAPTER 1

INTRODUCTION

Background and Rationale

Hematologic disease patients with thrombocytopenia often require frequent use of blood products, especially platelet concentrates, in order to prevent bleeding disorders and to support hemostatic competence. An inadequate post-transfusion response can occur in multi-transfused patients, which can result in life-threatening bleeding. Insufficient platelet increment after transfusion of suitable platelets is usually defined as platelet refractoriness. There are two major causes of decreased platelet increment; alloimmune and nonimmune.

Alloimmune causes involve antibodies that occur most frequently against the HLA class I and rarely against the human platelet antigens (HPA) system. In addition, platelet membranes also bear some antigens such as HLA class I molecules, human platelet specific antigens (HPA), and ABH, Lewis, P and I antigens. Antibodies to red cell antigens are also an alloimmune cause of platelet transfusion refractoriness.

Nonimmune causes are often associated with the patient's clinical conditions such as the presence of fever, sepsis, disseminated intravascular coagulation (DIC), splenomegaly, active bleeding and side effects of some drugs. Other disorders in which pathogenesis results are platelet alloimmunization, including neonatal alloimmune thrombocytopenia (NAIT) and post-transfusion purpura (PTP). Platelet alloantibodies cause immune-mediated platelet destruction because a variety of platelet-reactive antibodies react with antigens on platelet membranes. Platelets bearing immune complexes are destroyed by the reticuloendothelial (RE) system. These various disorders require a rapid diagnostic laboratory approach with sensitive and specific assays in order to provide appropriate therapy and to prevent long-term platelet destruction. Methods such as flow cytometry, solid phase red cell adherence assay (SPRCA), enzyme-linked immunosorbent assay (ELISA) and monoclonal antibody-specific immobilization of platelet antigens (MAIPA) are currently used for detection of platelet alloantibodies. These techniques are based on serological assays, however they have some limitations for routine application. Several

laboratories have struggled to find the best methods and provided necessary services for the related patients.

In Thailand, the National Blood Center uses the SPRCA technique for detection of HPA alloantibodies. This technique requires a longer time, is less sensitive and the reactions are read visually. Other techniques, however, are not well established in the other laboratories. The ideal test for antibody detection should be highly sensitive, very specific, rapid, requiring a small amount of serum, easy to perform at low cost and simple to interpret.

Platelet alloantibody detection techniques applied in routine laboratory use often posses some problems such as being complicated to perform, time consuming, and need expensive reagents and instruments. Evaluation and comparison of these techniques will provide benefits to laboratories through insights on which techniques are suitable for clinical services in particular environments. Blood transfusion services in a tertiary care hospital or a medical center need a high level of laboratory services in order to provide complicated patient care and treatment. The sensitivity and specificity of these various methods will be evaluated in order to establish the proper methods suitable for routine investigations of alloimmune causes of these disorders including platelet transfusion refractoriness, NAIT and PTP. This study will be very useful for the laboratories and beneficial to patients who will receive an accurate diagnosis and get better therapeutic outcomes.

Literature Review

A. Platelets

A.1 Platelet morphology and functions

Platelets or thrombocytes were discovered by Bizzozero in 1882 and then later recovered in the 1960's (Rozman, 2002). They are small anucleate fragments of bone marrow megakaryocytes approximately 1.3 – 3.3 μm in diameter, and mean platelet volume is about 10 femtoliter. In blood circulation, the normal number of platelets is about 150,000 to 400,000 per microliter, and a reduced platelet count is called thrombocytopenia. The number of platelets decreases when platelets are lost from the blood stream faster than they are replaced from the bone marrow. Thrombocytopenia may result from a failure of platelet production and/or an increased rate of platelet destruction. Their daily consumption ranges between 7-10 x 10^9 platelets per liter of blood and every second approximately 2 x 10^6 platelets are produced in normal human body. Average life span of platelets has been reported to be 7 to 10 days (Kickler, 2006). In healthy adults, about 30% of blood platelets are sequestered in spleen (Beutler et al, 2001).

The resting platelets have a discoid shape, and their morphology is divided into three zones: peripheral, sol-gel and organelle (Figure 1). The peripheral zone consists of a glycocalyx coat, cytoskeleton and platelet membrane responsible for adhesion and aggregation. The exterior coat of the platelet consists of at least nine different glycoproteins (GPIa, Ib, Ic, IIa, IIb, IIIa, IV, V, and IX) which are used for activation and interaction with other cells. The sol-gel zone contains the open canalicular system, the dense tubular system responsible for contraction. The organelle zone contains many types of chemicals such as ADP, serotonin, catecholamine, calcium, alpha granules, and glycogen granules. Since most of the common cytoplasmic organelles, including mitochondria, components of the Golgi apparatus, endoplasmic reticulum, and ribosomes are present in platelets, a platelet can be regarded as a structure potentially capable of all cellular functions except those immediately dependent on a nucleus (Han and Baker, 1964).

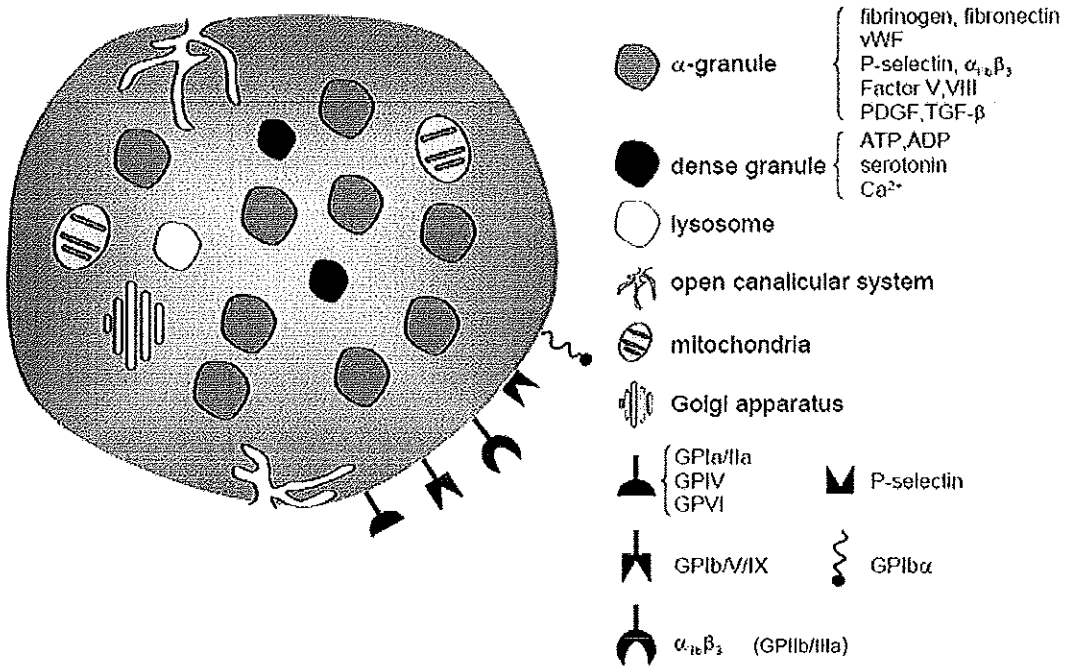


Figure 1. Schematic representation of platelet histology and membrane receptors (Nathan and Oski, 2003)

Platelets circulate in the blood stream and form blood clots to stop blood loss from injured blood vessels. They contribute to the hemostatic process in two different ways. Firstly, their adhesive and cohesive functions lead to the formation of platelet plugs to limit blood loss at sites of vascular injury. Secondly, they activate coagulation processes through the exposure of phospholipid surfaces that provide a catalytic site for the development of coagulation and stabilization of a hemostatic plug. A strong clot acts as a patch against blood leakage. Platelets manifest a triad of functional responses: adhesion, activation-secretion and aggregation. The process can be summarized in figure2. Within 1-3 seconds after an injury, glycoprotein (GP) Ib/IX/V complexes on platelet membranes bind to von Willebrand factor (vWF) on subendothelium, and GPIa/IIa complexes bind to collagen which then allow platelets to adhere to the injury sites. Platelet adhesion is shown in figure 3. A variety of platelet antagonists, including thrombin, epinephrine, collagen from subendothelium, and ADP, cause intracellular signaling

leading to secretion. Platelets secrete the dense granules which contain calcium, ADP, ATP, GTP, thromboxan A2 and serotonin. Alpha granules release adhesive molecules, fibrinogen, vWF, thrombospondin and fibronectin, which promote adhesion and aggregation. Secretion and shape changes leading to pseudopodia occur which facilitate aggregation to other platelets. Platelet aggregation is mediated by fibrinogen receptors (glycoprotein IIb/IIIa complexes), which bind fibrinogen and other adhesive proteins, forming the bridges that link platelets as shown in figure 4. All functional responses occur when extracellular signaling molecules are converted into intracellular chemical messengers through the platelet membrane receptors and glycoproteins.

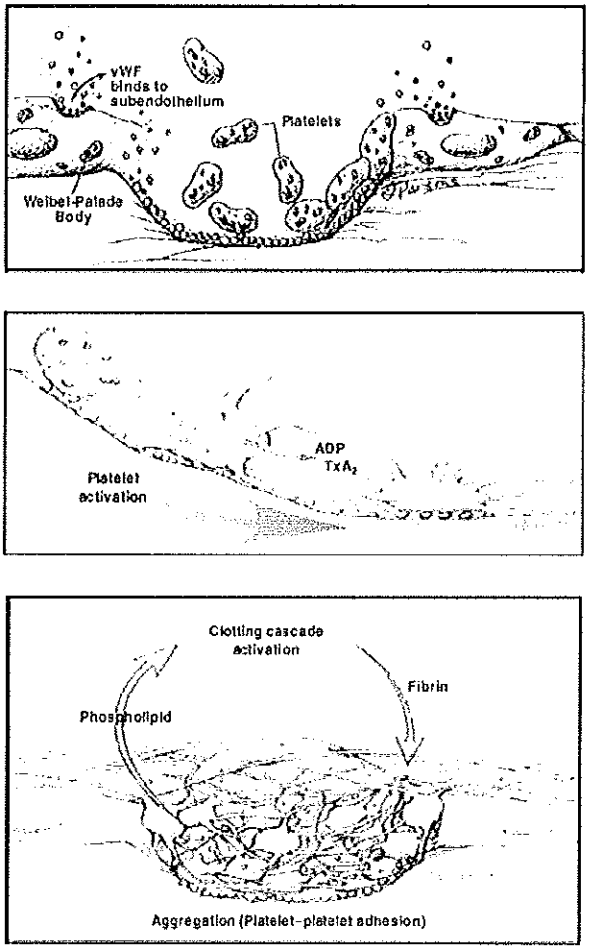


Figure 2. Overview of the three main platelet functions of adhesion, secretion and aggregation (Kickler, 2006)

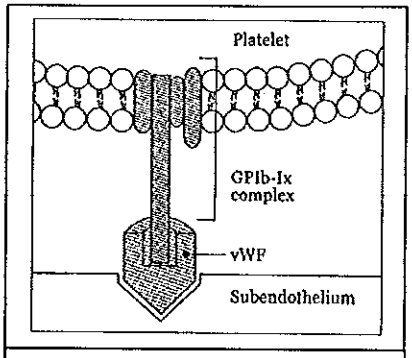


Figure 3. Overview of platelet adhesion involving platelet glycoprotein receptor (GPIb-IX) and von Willebrand factor (Kickler, 2006)

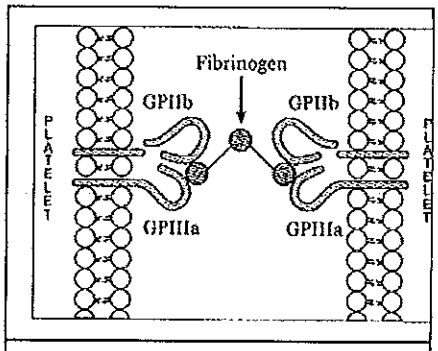


Figure 4. Overview of platelet aggregation to other platelets by fibrinogen binding to platelet GPIIb/IIIa (Kickler, 2006)

A.2 Platelet alloantigens

There are several antigens on a platelet's surface, and some of these antigens are shared by other cell types. More than 45 different membrane structures are found on resting platelets and can be divided into five groups: adhesion molecules, immune molecules, receptors, blood group antigens and some other molecules (Rozman, 2002). There are two groups of clinical relevant platelet alloantigens: platelet-nonspecific alloantigens (blood group antigens and HLA class I antigens) and platelet-specific alloantigens.

A.2.1 Blood group antigens

Platelet membranes bear ABO and some other polysaccharide blood group antigens such as Le^a, Le^b, Ii, and P. ABH antigens occur on platelets in two types: extrinsic determinants representing glycosphingolipids adsorbed from plasma, and intrinsic determinants carried predominantly by glycolipids and a variety of glycoproteins. The amounts of ABH antigen present are quite variable from individual to individual (Mueller Eckhardt, 1990). The antigen density is much weaker than antigen density appears on red cells (about 5%). The survival of

transfused platelets may be affected by anti-A and anti-B. ABH antigens may be the cause of the reduced survival of ABO-incompatible platelets transfusion, and as a result occasional cases may become refractory. However, A1 antigens on platelets play a minor role in platelet transfusion because in rare cases refractory to A1 platelets have been described in group O patients with strong A1-specific IgG antibodies (Friedgord, 1999). In addition, there is no evidence that antibodies to other blood group antigens significantly reduce platelet survival *in vivo* (AABB, 1999).

A.2.2 HLA class I antigens

The major histocompatibility complex (MHC) in humans, also known as the human leukocyte antigen (HLA), is a large genomic region of highly polymorphic genes. The HLA region is located on chromosome 6p21.1 to p21.3, with class II, class III and class I genes found from the centromeric to the telomeric end respectively. This set of genes is known to play a key role in the immune system, autoimmunity, and reproductive success. The HLA class I and HLA class II regions contain genes that encode cell-surface glycoproteins. One of the major roles of their products in the physiological immune response involves the antigen presentation to T cells. The class I region encodes HLA class I molecules, which are expressed on the cell surface of nucleated cells and are involved in the presence of peptides predominantly derived from intracellular proteins to CD8+ cytotoxic T cells (CTLs). The class II region encodes many membrane-bound proteins expressed on the cell surface of B lymphocytes, macrophages, monocytes, dendritic cells and endothelial cells that interact exclusively with CD4+ helper T cells. HLA class I antigens are highly polymorphic and categorized as HLA-A, -B and -C antigens. Platelet membranes also bear HLA class I molecules shared by platelets and white blood cells. The expression of HLA class I antigens on platelets can vary substantially among individuals and among antigens. The amounts of HLA-A or HLA-B antigen are at least ten fold higher than HLA-C antigen. This would account for the low immunogenicity of platelet HLA-C antigens, making it unnecessary to match HLA-C locus antigens for donor-recipient compatibility in platelet transfusion (Mueller Eckhardt, 1980). HLA class I antigens are the main intrinsic determinants of platelets present with approximately 100,000 copies per platelet. However, soluble HLA antigens

can be taken up by human platelets from plasma in small amounts (Santoso, 1986). Antibodies react with HLA class I antigens on platelet surfaces result in platelet refractoriness.

A.2.3 Platelet-specific antigens

Platelet-specific antigens located on platelet-membrane glycoproteins (GP), are classified into the following complexes: GPIa/IIa (the collagen receptor), GPIc/IIa (the fibronectin receptor), GPIc'/IIa(the laminin receptor), GPIb/ IX/ V (the vWF receptor), GPIIb/IIIa (the fibrinogen receptor). These glycoproteins play an important role in the platelet's immunology and functions. Platelet-specific alloantigens are polymorphic epitopes of platelet membrane glycoproteins. Most of these polymorphisms were found by the clinical observation of alloantibodies that were found following immunization by blood transfusion, pregnancy or transplantation (Santoso et al, 1998).

A.2.3.1 Nomenclature and molecular genetics of human platelet alloantigens

Initially, platelet-specific alloantigens were named in a group based on the name of patients who developed platelet alloantibodies. The first platelet-specific alloantigen was Zw found by van Loghem et al in 1959 in a patient with post transfusion purpura. In 1989, Newman et al discovered the Zw polymorphism due to a single amino acid substitution at position 196 of the GPIIIa, and gave the name $Zw^{a/b}$. Later, this nomenclature became a problem since multiple designations existed for the identical antigens due to independently discovered alloantisera, such as Zw and PI in table 1. To solve this problem and to avoid confusion a new nomenclature was proposed by International Society of Blood Transfusion (ISBT) in 1990 (von dem Borne et al., 1990). The platelet-specific alloantigen systems are called HPA for Human Platelet Antigen. The different antigen systems are numbered chronologically according to the date of publication. The allelic antigens are designated alphabetically according to their frequency in the population, the high-incidence allele first (=a) and the lower-incidence allele second (=b). The inclusion of the new HPA systems will need the approval of the working party.

In 2003 the ISBT platelet working party and the International Society on

Thrombosis and Haemostasis (ISTH) created the Platelet Nomenclature Committee (PMN). The PMN committee added some new rules to the HPA system: a platelet-specific antigen is called HPA when its molecular basis has been defined. A designation "w" is added after the antigen name if an alloantibody against the antithetical antigen has not been reported. Moreover, there are two groups of serologically defined platelet-specific antigens that have been published, but their genetic base has not yet been determined (table 2). To date, 24 platelet-specific alloantigens have been defined by immune sera and are shown in tables 3 and 4. Only 12 of them are grouped in six biallelic systems (HPA-1a1b,-2a2b,-3a3b,-4a4b,-5a5b,-15a15b). For the remaining 12, alloantibodies against only the thetical antigen have been observed (Metcalfe et al., 2003 and Panagiota, 2004).

In all but one of the 22 antigens a single amino acid substitution caused by a single nucleotide polymorphism (SNP) in the gene encoding for the relevant membrane glycoprotein defined the difference of the immunogenetic epitope of HPA (table 3). These antigens are polymorphic, and they can induce production of alloimmune antibodies.

Table 1. Human platelet antigens (Metcalfe et al., 2003)

System	Antigen	Original names	Glycoprotein location	CD
HPA-1	HPA-1a	Zw^a, Pl^{A1}	GPIIIa	CD61
	HPA-1b	Zw^b, Pl^{A2}	GPIIIa	CD61
HPA-2	HPA-2a	Ko^b	GPIbα	CD42b
	HPA-2b	Ko^a, Sib^a	GPIbα	CD42b
HPA-3	HPA-3a	Bak^a,L ek^a	GPIIb	CD41
	HPA-3b	Bak^b	GPIIb	CD41
HPA-4	HPA-4a	Yuk^b, Pen^a	GPIIIa	CD61
	HPA-4b	Yuk^a, Pen^b	GPIIIa	CD61
HPA-5	HPA-5a	Br^b, Zav^b	GPIa	CD49b
	HPA-5b	Br^a, Zav^a, Hc^a	GPIa	CD49b

Table 1. Human platelet antigens (Metcalfe et al., 2003) (continued)

System	Antigen	Original names	Glycoprotein location	CD
	HPA-6bw	Ca[a], Tu[a]	GPIIIa	CD61
	HPA-7bw	Mo[a]	GPIIIa	CD61
	HPA-8bw	Sr[a]	GPIIIa	CD61
	HPA-9bw	Max[a]	GPIIb	CD41
	HPA-10bw	La[a]	GPIIIa	CD61
	HPA-11bw	Gro[a]	GPIIIa	CD61
	HPA-12bw	Iy[a]	GPIbβ	CD42c
	HPA-13bw	Sit[a]	GPIa	CD49b
	HPA-14bw	Oc[a]	GPIIIa	CD61
HPA-15	HPA-15a	Gov[b]	CD109	CD109
	HPA-15b	Gov[a]	CD109	CD109
	HPA-16bw	Duv[a]	GPIIIa	CD61

Table 2. Platelet antigens without HPA assignment (Metcalfe et al., 2003)

Antigen	Glycoprotein
Va[a]	GPIIb/IIIa
Mou[a]	Unknown

Table 3. Polymorphisms resulting in human platelet antigens (HPAs) (Metcalfe et al., 2003)

Antigen	Glycoprotein	Nucleotide change	Amino acid substitution
HPA-1	GPIIIa	176 T>C	L33P
HPA-2	GPIbα	482C>T	T145M
HPA-3	GPIIb	2621T>G	I843S
HPA-4	GPIIIa	506G>A	R169O
HPA-5	GPIa	1600G>A	E534K
HPA-6w	GPIIIa	1544G>A	R515Q
HPA-7w	GPIIIa	1297C>G	P433A
HPA-8w	GPIIIa	1984C>T	R662C
HPA-9w	GPIIb	2602G>A	V868M
HPA-10w	GPIIIa	263G>A	R88Q
HPA-11w	GPIIIa	1976G>A	R659H
HPA-12w	GPIbβ	119G>A	G40E
HPA-13w	GPIa	2483C>T	T828M
HPA-14w	GPIIIa	1909-11delAAG	K637del
HPA-15	CD109	2108C>A	S703Y
HPA-16w	GPIIIa	497C>T	T166I

A.2.3.2 Polymorphisms of the glycoprotein complexes

It has been shown that all platelet-specific antigens are localized on platelet membrane glycoproteins such as GPIIb, GPIIIa, GPIb, GPIa and CD109. Platelet glycoprotein polymorphisms resulted from a single nucleotide, and the resulting amino acid changing, but no functional defects were seen. GPIIb/IIIa complex is a heterodimeric integrin consisting of approximately 50,000-80,000 copies of the heterodimer per platelet. The gene encoding GPIIb and IIIa, *GP2B** and *GP3A**, are located on the long arm of chromosome 17. The genetic basis of 11

alloantigens on the complex is documented (HPA-1, -3, -4, -6w, -7w, 8w, -9w, -10w, -11w, -14w, -16w) and 10 of them involve single nucleotide substitutions. One exception, the HPA-14bw antigen, is the result of an "in-frame" deletion of three nucleotides. The GPIb/V/IX complex is composed of four transmembrane components: GPIbα and GPIbβ, which are linked by a disulfide bond and non-covalently associated with GPIX and GPV, the other two. There are approximately 25,000 copies of GPIb/IX and 12,000 copies of GPV per platelet. The chromosomal location of the GPIbα gene (*GP1BA**), is on chromosome 17, the GPIbβ gene (*GP1BB**) is on chromosome 22 and the GPV and GPIX (*GP5* and GP9**) genes are on chromosome3. The GPIbα gene has two allelic forms that result in the formation of HPA-2a and HPA-2b epitopes. HPA-12 alloepitope is expressed on the GPIbβ. The other two GP of this complex, GPV and GPIX, are not polymorphic (Rozman, 2002). The GPIa/IIa complex is another platelet membrane integrin. There are approximately 800-2,800 copies of GPIa/IIa per platelet, and only GPIa is polymorphic. Gene encoding for GPIa (*GP1A*) is located on chromosome 5 and gives rise to the HPA-5 and HPA-13 alloepitopes on the GPIa (Rozman, 2002).

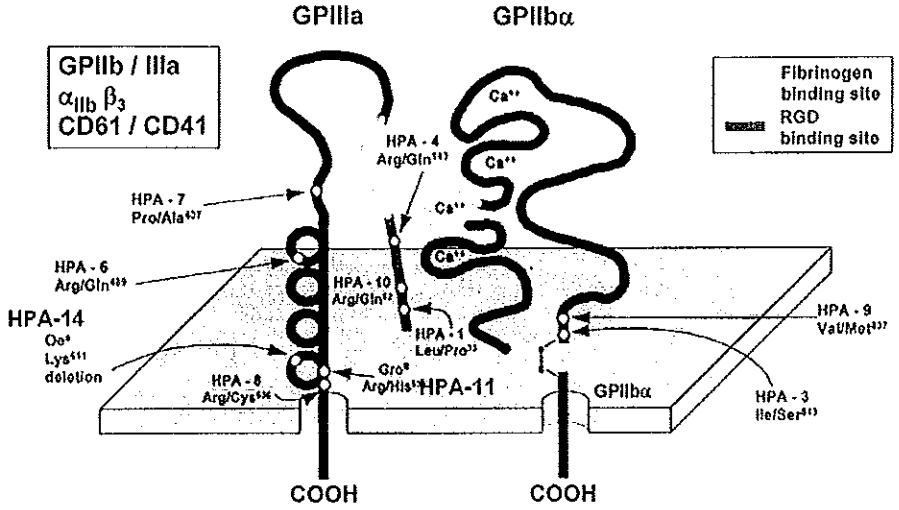


Figure 5. Schematic representation of the amino acid substitutions which determines the various antigens on GPIIb/IIIa, based on the illustration published by Bachelot et al., 1995

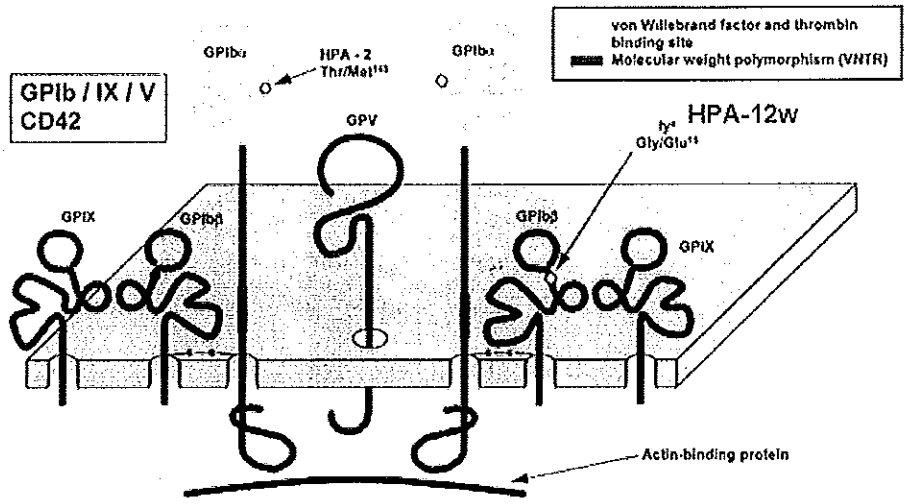


Figure 6. Schematic representation of the amino acid substitutions which determines the various antigens on GPIb/IX/V, based on the illustration published by Bachelot et al., 1995

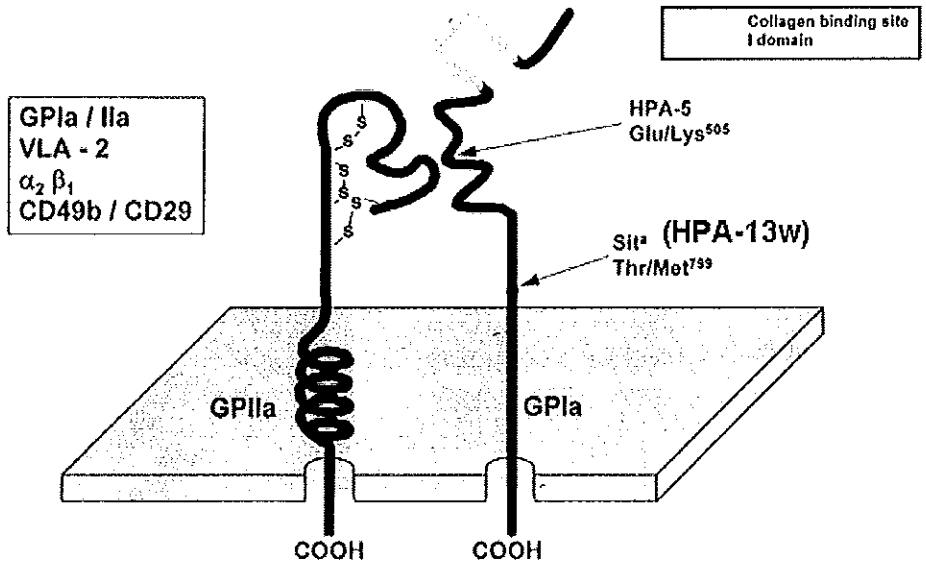


Figure 7. Schematic representation of the amino acid substitutions which determines the various antigens on GPIa/IIa, based on the illustration published by Bachelot et al, 1995

A.2.3.3 HPA typing

HPA typing of blood donors is not routinely performed. Usually, ABO-matched platelets are transfused. HPA typing is essential in several different clinical conditions and blood services. In situations where the recipient has HPA antibodies resulting in refractoriness to platelet transfusions, neonatal alloimmune thrombocytopenia (NAIT) and post-transfusion purpura (PTP) HPA-typed platelets may be needed. For the study of HPA polymorphisms in the population accurate HPA typing is required. In recent years HPA typing methods have been established and can be divided into two techniques: serological HPA phenotyping and HPA genotyping by molecular methods. Serological techniques for HPA phenotyping based on the principle of indirect immunofluorescent assay, mixed passive hemagglutination and ELISA. The value of serological typing is limited since often too few platelets can be obtained from thrombocytopenic patients, and reliable serotyping reagents for HPA antigens are rare. Serum often contains both HPA and HLA class I antibodies. However, monoclonal antibodies are now applied in phenotyping through such methods as the monoclonal immobilization of platelet antigen assay (MAIPA). These phenotyping assays can complement genotyping assays for the provision of a HPA-selected donor.

For the genotyping of most important HPA epitopes which are located on the GPIIIa, GPIbα, GPIIb, and GPIa, several techniques of polymerase chain reaction (PCR) have been implemented. HPA genotyping can be carried out with genomic DNA extracted from blood or any suitable cellular materials, and commercially available reagents are used. Various applications of the PCR-based method for HPA genotyping were described, such as restriction fragment length polymorphism analysis (RLFP), allele-specific oligonucleotide hybridization, and polymerase chain reaction with sequence-specific primer (PCR-SSP). Among these techniques PCR-SSP is the most widely used (Panzer, 2001). PCR-SSP has been adopted by many laboratories because of its simplicity, and protocols that allow the determination of several HPA genotypes under the same PCR conditions have been described.

A.2.3.4 Frequencies of HPA in different populations

There are many studies which have reported the frequency of platelet-alloantigens in different populations. Phenotype and genotype frequencies have been determined by a variety of serological and biomolecular methods and they are summarized in tables 4 and 5. The data clearly show significant differences in the prevalence of some antigens between populations. DNA-base typing has confirmed earlier serological showing that there is a variation in HPA allele frequencies between various Caucasian, Asian, African and African American populations. In Caucasian, "b" variants of HPA-1, -2, -3, and -5 alloepitopes occur more often, while HPA-4 is found in the homozygous form a/a in more than 99.9% of the Caucasian population. HPA-1b is rare in Asians, while the HPA-4b variant is more common than that in Caucasians. In the Thai population gene frequencies of HPA were studied by using PCR-SSP. The study showed that the most common distribution of HPA were HPA-1a, -2a, -4a, -5a and -6a, where as HPA-1b, -2b, 4b, -5b and -6b were rarely found. The gene frequencies of HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6a and -6b were 98.5%, 1.5%, 95.2%, 4.8%, 56.0%, 44.0%, 100.0%, 0.0%, 96.8%, 3.2%, 98.6% and 1.4%, respectively (Kupatawintu et al., 2005). The frequencies of HPA in Thais are similar to those in other Asian populations, but some antigens are different from those in white populations (Shih et al., 2003). A recent study of the UK population using PCR-SSP in 134 subjects found the gene frequencies of HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, -5b, -6a and -6b were 84.0%, 16.0%, 92.5%, 7.5%, 62.7%, 37.3%, 100.0%, 0.0%, 91.4%, 8.6%, 100.0% and 0.0%, respectively. Several studies have shown that there are higher gene frequencies of HPA-1b, -2b and -5b in Caucasians than those in Asian populations (Jone et al., 2003). Similar to Caucasians, Brazilians and Africans show higher gene frequencies of HPA-1b, -2b and -5b than those in Asians. In Brazilians gene frequencies of HPA-1a, -1b, -2a, -2b, -3a, -3b, -4a, -4b, -5a, and-5b, were 91.8%, 8.2%, 86.1%, 13.9%, 64.0%, 36.0%, 100.0%, 0.0%, 82.5% and 17.5%, respectively (Chiba et al., 2000). Data on the prevalence of HPA antigens in a given population are essential for the diagnosis of immune thrombocytopenias, planning of screening programs for women at risk of neonatal alloimmune thrombocytopenia, genetic counseling and provision of therapy for patients with anti-HPA antibodies (Mueller Eckhardt et al., 1989). Moreover, HPA distribution among various populations is important for the prediction of the risk of platelet-specific alloimmunization

in different ethnic groups and for the ability to prepare HPA compatible platelet products for patients.

Table 4. Gene frequencies of HPA-1 to - 6 in various populations and in Thais (Kulkarni et al., 2005 and Kupatawintu et al., 2005)

HPA polymorphism	Antigen	U.S. Africans (Drezewek et al.,1998)	Caucasian (Chiba et al., 2000)	Brazil (Chiba et al., 2000)	Japanese (Tanaka et al.,1996)	Thai (Kupatawintu et al., 2005)
HPA-1	HPA-1a	92.0	85.0	91.8	>99.0	98.5
	HPA-1b	8.0	15.0	8.2	<1.0	1.5
HPA-2	HPA-2a	82.0	93.0	86.1	87.9	95.2
	HPA-2b	18.0	7.0	13.9	12.1	4.8
HPA-3	HPA-3a	63.0	61.0	64.0	55.5	56.0
	HPA-3b	37.0	39.0	36.0	44.5	44.0
HPA-4	HPA-4a	100	>99.9	100	99.0	100.0
	HPA-4b	0	<0.1	0	1.0	0.0
HPA-5	HPA-5a	79.0	89.0	82.5	95.4	96.8
	HPA-5b	21.0	11.0	17.5	4.6	3.2
HPA-6	HPA-6a	-	>99.7	-	97.7	98.6
	HPA-6b	-	<0.3	-	2.3	1.4

Table 5. Gene frequencies of HPA-1 to -6 in Thai populations from different regions. (Kongmaoeng et al., 2001; Shih et al., 2003; Romphruk et al., 2000 and Kupatawintu et al., 2005)

Ethnic HPA polymorphism	Antigen	Central Thai N=355 (Kongmaoeng et al., 2001)	Thai N=137 (M.C.Shih. et al., 2003)	North-Eastern Thai N=292 (Romphruk et al., 2000)	Thai blood donors N=500 (Kupatawintu et al., 2005)
HPA-1	HPA-1a	98.31	98.50	97.26	98.5
	HPA-1b	1.69	1.50	2.74	1.5
HPA-2	HPA-2a	96.20	93.8	93.66	95.2
	HPA-2b	3.80	6.20	6.34	4.8
HPA-3	HPA-3a	56.34	50.70	53.60	56.0
	HPA-3b	43.66	49.30	46.40	44.0
HPA-4	HPA-4a	99.72	100	100	100.0
	HPA-4b	0.28	0	0	0.0
HPA-5	HPA-5a	96.90	96.30	96.23	96.8
	HPA-5b	3.10	3.70	3.76	3.2
HPA-6	HPA-6a	98.31	98.50	98.46	98.6
	HPA-6b	1.69	1.50	1.54	1.4

A.3 Antibodies against platelet alloantigens

A.3.1 The mechanism of alloimmunization to HPA alloepitopes

HPA molecules on platelet glycoproteins can induce alloimmunization of the recipient in the general patterns of alloimmune response. There are two possibilities for alloimmunization pathways that result in the formation of platelet alloantibodies. The first

pathway occurs when the recipient T cell receptor (TCR) on the CD4+ T cells directly interacts with the foreign HPA alloepitope, which is presented in a groove of the MHC class II molecule on the surface of the donor antigen presenting cells (APC). The second mechanism is an indirect way which involves the processing of foreign donor antigens by the host APC. The allogeneic donor peptide within the MHC-class II molecules on the APC is presented to the recipient CD4+ T cells. In both pathways adequate signaling is required for the CD4+ T cell to become activated and secrete cytokines such as interleukins-2 and interferon-gamma. These cytokines can stimulate antigen-prime Bcells to differentiate into plasma cells and produce antibodies. It is believed that the indirect pathway is probably responsible for the majority of the IgG immune responses to antigens derived from red cells, platelets and plasma proteins (Semple et al., 1999).

A.3.2 Conditions associated with platelet antibodies and incidences in various populations

Immune-mediated platelet destruction disorders which are clinical problems result from a variety of platelet-reactive antibodies such as neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP) and platelet refractoriness.

A.3.2.1 Neonatal alloimmune thrombocytopenia (NAIT)

NAIT, often known as feto-maternal alloimmune thrombocytopenia (FMAIT), is caused by maternal immunoglobulin-G (IgG) alloantibodies, which cross the placenta and are directed against paternal alloantigen on fetal platelets. The mother produces an antibody-mediated response against a platelet-specific antigen that she herself lacks but that is present on fetal plateletshaving been inherited from the father. The mechanism is similar to hemolytic disease of the newborn (HDN), except that approximately 30-50% of cases occur in a severe form in the first pregnancy, and subsequent pregnancies are usually affected (Metcalfe, 2004 and Eline, 2008). The clinical manifestations are variable, ranging from asymptomatic to severe intracranial hemorrhaging and death. Some studies showed a high risk of severe NAIT being correlated with high antibody levels (Taegtvik et al., 2000 and Bertrand et al., 2006). In white populations, NAIT occurs in approximately 1 out of 1,000 to 1 out of 2,000 neonates (Adulgabar, 2003). About 85%

of NAIT cases are caused by anti-HPA-1a, 5-15% by anti-HPA-5b and 5% by other antibodies. Approximately 1.5-2.0% of pregnant Caucasian women are HPA-1a negative (Eline, 2008). In Asian populations alloimmunization to HPA-1a is rare, and some case reports of NAIT are caused by anti-HPA-4a. Among Asians gene frequency for HPA-1b is lower than in Caucasian populations (<0.1% vs 1.5%). Therefore, anti-HPA-1a has not been shown to cause NAIT in Asian populations (Nago et al., 1991).

It has been suggested that NAIT can be caused by anti-HLA class I antibodies, but this is controversial (Kyou-Sup et al., 2002 and Harrison et al., 2003). Because platelets also carry ABH antigens, the blood group A and B IgG alloantibodies may cause NAIT (Tagning et al., 1994). In a case report by del Rosario et al in 1998 maternal anti-HLA antibodies were found in the serum of a new born during the thrombocytopenic period. These authors suggested a potential pathogenetic role of maternal anti-HLA antibodies in NAIT (del Rosario et al., 1998). In another report Skacel et al. studied 147 maternal sera. They found that in 39% of the maternal sera anti-HLA antibodies were demonstrated, but no associations with decreased platelet or granulocyte counts in the corresponding infants were recorded (Skacel et al., 1989).

The diagnosis of NAIT is based on a serology analysis. Moreover, maternal-fetal HPA incompatibility can be confirmed by paternal HPA typing. In cases where the father is homozygous for the specific HPA antigen one can assume the fetus is at risk. In cases where the father is heterozygous for HPA antigen amniocentesis is currently used for fetal HPA typing. Therefore, all pregnancies in which the mother carries HPA antibodies and the fetus is positive for the corresponding HPA antigen must be regarded as at risk for NAIT (Eline, 2008).

A.3.2.2 Post-transfusion purpura (PTP)

Post-transfusion purpura (PTP) is a rare but severe bleeding disorder usually occurring 7-10 days after the transfusion of blood components. It is characterized by an acute thrombocytopenia and platelet counts below 15,000 per microliter leading to life-threatening bleeding complications without intervention (Lucas, 1997 and Woelke, 2006). About 150-200 cases of PTP have been reported in the literature (Mc Farland, 2001). The estimation of its incidence suggests that it occurs in 1:50,000 to 100,000 transfusions. It has rarely been reported in

men, and the female to male ratio is >20:1 (Muller Echardt, 1990).

All PTP patients have had previous exposure to allogeneic platelets through pregnancy or blood transfusion. Over 90% of cases in Caucasian populations are due to anti-HPA-1a. In rare cases platelet-reactive antibodies other than anti-HPA-1a, such as HPA-3a or -5b, were reported (von dem Borne et al., 1980, Lubenow et al., 2006).

The pathogenesis is unclear. The most likely explanation is the production of cross-reactive alloantibodies in the early phase of the anamnestic response. The patients are sensitized by a previous pregnancy or transfusion and respond to a second challenge of incompatible platelets by making high-titer HPA (and often HLA) antibodies. The resulting immune destruction of transfused platelets may contribute to the simultaneous destruction of the patient's own platelets, but the exact mechanism is not clear. Two hypotheses have been proposed. The destruction of transfused platelets may release alloantigen which is then adsorbed onto the surface of the patient's own platelets, followed by the attachment of alloantibodies which are consequently were removed from the circulation. Another possibility is the simultaneous, but short–lived, formation of autoantibodies. However, these mechanisms can not explain pathogenesis in all cases of PTP patients. When thrombocytopenia occurs in the patient platelet transfusion is generally not recommended because both random and HPA-compatible platelets are usually ineffective in achieving increments. Most patients respond to high doses of intravenous immunoglobulins (IVIG). In a few cases spontaneous recovery occurs within 1-4 weeks of the onset of thrombocytopenia (Metcalfe, 2004).

A.3.2.3 Platelet transfusion refractoriness (PTR)

Transfusion support with platelet concentrates (PCs) is widely applied and accepted for the prevention and treatment of bleeding in patients with thrombocytopenia. The major problem associated with long-term platelet transfusions is an inadequate post-transfusion increment, which can result in life-threatening bleeding. Failure to obtain an adequate increase in platelet count occurs in about 20-70% of multitransfused thrombocytopenic patients. Insufficient platelet increment after the transfusion of suitable platelets is usually defined as platelet

transfusion refractoriness. There is not a strong consensus in the criteria for determining platelet refractoriness. The most commonly accepted definition of platelet refractoriness is defined by the American Society of Clinical Oncology. It is defined as a lack of expected platelet increments following two consecutive platelet transfusions (Schiffer et al., 2001). Post-platelet transfusion response is often determined by calculating a corrected count increment (CCI) as follows:

$$CCI = \frac{\text{(post-transfusion platelet count – pre-transfusion platelet count) X body surface area } (m^2)}{\text{Transfused platelets } (10^{11})}$$

If CCI at 10 to 60 minutes and at 18 to 24 hours after the transfusion are below 7,500 and 4,500 per microliter respectively, the patient is considered to have platelet refractoriness (Contreras et al., 1998).

Many factors are involved in platelet transfusion refractoriness. Approximately 24-44 percent of refractoriness in patients is associated with clinical or pharmacological causes (non-immune causes), and in some cases refractoriness is caused by immune factors. The most common immunological refractoriness is caused by alloimmunization against HLA class I antigens. Thirty to seventy percent of patients receiving multiple transfusions develop HLA alloantibodies, but only 8 to 40 percent of these immunized patients have platelet refractoriness (Panagiota, 2004). The Trial to Reduce Alloimmunization to Platelets (TRAP) study showed that 45% of the immunized patients (either by transfusion or pregnancy) developed anti-HLA antibodies, but only 13% of this group had platelet refractoriness. There is no dose-response relationship between the number of platelets transfused and the incidence of alloimmunization (Schiffer et al., 1976; Dutcher et al. 1981; Hod and Schwartz, 2008). Hod and Schwartz reviewed several studies on platelet refractoriness and suggested that alloimmunization and refractoriness were complicated processes with unknown modified factors determining whether an individual will become alloimmunised and whether this alloimmunization will cause refractoriness to transfusion (Hod and Schwartz, 2008).

The incidence of platelet-specific antibodies varies from 2 to 10% in Caucasians (Mueller-Ekchardt, 1990; Kiefel et al., 2001; Sanz et al., 2001 and Panagiota, 2004). In a study by Kiefel et al. (2001) platelet-specific antibodies were detected in the sera of 8% of multi-transfused

Caucasian patients. The main platelet alloantibody specificities were anti-HPA-5b and -1b where as anti-HPA-1a was the most prevalent in NAIT and PTP. In a study by Sanz et al. (2001) only 3.8% of HLA alloimmunised patients had platelet-specific antibodies and the specificity observed was anti-HPA-5b. In Thailand Kupatawintu et al. (2000) studied 163 thrombocytopenic patients and reported that HPA antibodies were detected in 6.13% of the cases studied. The main platelet alloantibody specificities were anti-HPA-5b and -2b. There was a study which reported that 17 patients were found to have platelet-specific antibodies wich were not present at registration, 12 patients (70 %) first developed them during a period of infection and it was usually transient (McGrath et al., 1988). To differentiate platelet refractoriness caused by alloimmune or non-alloimmune factors, many tests are used in both routine testing and research laboratories.

B. Techniques used for platelet alloantibodies detection

Unlike easily detected agglutination and lysis endpoints used in red blood cell immunological assays, detection of platelet-reactive antibodies requires techniques to measure direct interaction of immunoglobulins with platelets. Several antibody-detection methods have been reported, such as tests based on platelet function endpoint, assays that detect platelet associated immunoglobulins and those that detect the binding of antibodies to alloantigens located on isolated platelet surface glycoproteins. The earliest methods involved mixing patient serum with normal platelets and measured platelet function-dependent endpoints such as alpha granules release, aggregation or agglutination. In 1961, van der Weerdit et al. proposed a platelet agglutination test. However, these techniques have low sensitivity and specificity. The platelet immunofluorescene test (PIFT), mixed passive hemagglutination (MPHA) and enzyme-linked immunosorbent assay (ELISA) are the techniques used to detect platelet associated immunoglobulins that provide improved sensitivity in the detection of platelet alloantibodies (Shibata et al, 1981 and Levin et al, 2003). PIFT, a slide method that uses fluorescent-labeled anti-humanglobulin, was first introduced by van dem Borne et al. in 1978. PIFT by flow cytometry was applied by Kohler et al. in 1995. In 1981, Shibata et al used MPHA to detect and identify platelet alloantibodies. ELISA is another technique used to detect platelet associated immunoglobulins. It was described by Horai S et al., and was improved by Schiffer CA et al in 1983. Flow cytometry,

MPHA and ELISA are useful in platelet crossmatching for patient refractory to platelet transfusion. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA), an assay that detects antibodies of isolated platelet glycoproteins, was first demonstrated by Kiefel et al. in 1987. MAIPA requires murine monoclonal antibodies that recognize the target antigens, but do not compete with the human antibodies being detected.

B.1 Flow cytometry

The principles of the flow cytometry technique, briefly explained, are that platelets are sensitized with patient's sera. Free antibodies are removed, and platelet-bound antibodies are detected with a fluorescent-labeled (fluorescein isothiocyanate, FITC) monoclonal antibody specific for human immunoglobulin. The platelets are analyzed with a flow cytometer. The result can be expressed as the ratio of fluorescence of normal platelet sensitized with test serum over normal platelets incubated in a control serum.

It has been shown by several groups that platelet-reactive antibodies, including platelet crossmatching, can be detected through flow cytometric analysis of intact platelets (Worfolk et al., 1991; Skogen et al., 1995 and Kohler et al., 1996). Kohler et al. (1996) studied for the sensitivity and specificity of flow cytometric detection of platelet-reactive antibodies when MAIPA assay was taken as a reference. They found that the flow cytometry technique had nearly the same sensitivity and specificity as MAIPA, 94.7% and 96.3%, respectively. Moreover, the results of MAIPA and flow cytometry in detection of platelet-reactive antibodies correlated well ($p<0.0001$, $r=0.84$) (Kohler et al., 1996). This was also observed in two U.K. national platelet workshops where flow cytometry was more sensitive than microscopic detection but appeared to be less sensitive than the MAIPA assay (Allen et al., 1994). Worfolk et al. demonstrated that flow cytometry was sensitive, it able to detect as few as 1,000 molecules of bound fluorescein per cell under optimal conditions. In addition to platelet antibody screening, flow cytometry has proven useful in platelet crossmatching, with good sensitivity, specificity and predictive value (Worfolk et al., 1991). Freedman et al. compared LCT and SPCA with flow cytometry technique in platelet crossmatching and found that flow cytometry technique was the best sensitivity and specificity (Freedman et al., 1996). Levin et al showed LCT and flow cytometry technique were significantly

correlated in 189 samples (Levin et al., 2003).

Although there is a requirement for expensive flow cytometer, the flow cytometric approach is simple, rapid and allows to simultaneous detection of either HLA or HPA antibodies. The results are objective and semiquantitative. The fact that it is capable of distinction between HLA and platelet-specific antibodies makes it potentially suitable for either platelet antibody screening or platelet crossmatching.

B.2 Solid phase red cell adherence assay (SPRCA)

SPRCA is based on the principle of the mixed passive hemagglutination assay (MPHA). In this assay, platelet screening cells are immobilized in wells of a microplate, and then incubated with patient and control sera. Indicator red cells that are coated with human immunoglobulin are used to determine the reactions. If antibodies bind to immobilized platelets, hemagglutination is considered to be present. In negative reactions a button of red cells forms in the center of the well. Solid-phase platelets can be treated with chloroquine for elution of HLA class I antigens from platelet surface resulting in only platelet-specific antibodies being detected (Lown and Ivey, 1991).

Shibata et al reported a passive haemagglutination method to detect platelet antibodies and to crossmatch using platelets bound to microplates and anti-IgG-coated sheep red cells as the indicator (Shibata et al., 1981). Lown and Ivey evaluated SPRCA parallel with the platelet suspension imunofluorescence test (PIFT) by microscopy and found the SPRCA technique displayed similar sensitivity and specificity to the PIFT. They recommended for use by routine hospital laboratories to screen platelet antibodies (Lown and Ivey 1991). Shibata studied in 252 Japanese patients receiving multiple platelet transfusions and they found alloimmunisation in 2.6% by LCT method. By the SPRCA, the positive rate was 5.9%. The higher positive rate identified by SPRCA was explained by its higher specificity compared with the LCT (International Forum, 2003). In Thailand SPRCA is commonly used to detect platelet-reactive antibodies and to apply in platelet transfusion. The speed and simplicity of SPRCA allow many hospital laboratories to use it for platelet antibody screening and crossmatching. The major drawbacks of SPRCA are its insensitivity and subjective results.

B.3 Antigen capture ELISA (ACE)

ACE is a solid phase ELISA used to detect antibodies to platelet glycoproteins. The principles of this method are as follows. Microwells are coated with platelet glycoproteins, serum samples are incubated, and unbound immunoglobulins are removed. Alkaline phosphatase labeled anti-humanglobulin reacts with the substrate and develops color in the case of a positive reaction. The reaction stops and measurement of the absorbance (optical density, OD) is performed. The results are expressed by comparison of the OD of test with the negative cut off.

An advantage of antigen capture techniques is the ability to exclude non-platelet specific reactivity. Use of panels of platelets with known alloantigen phenotypes allowed the identification of platelet antibody specificities in the evaluation of platelet alloimmune disorders. Disadvantages of ACE are technically demanding to perform, requiring experienced technologists. A further potential disadvantage of antigen capture assay is the possible disruption of alloantibody epitopes by detergent solubilization (Mc Farland, 2003).

B.4 Monoclonal antibody-specific immobilization of platelet antigens (MAIPA)

MAIPA is performed according to Kiefel et al with some modifications. Target platelets are incubated with patient serum, washed, and then murine-monoclonal antibodies against GPIIb/IIIa, GPIb/IX, and HLA class I antigens are added. After the initial sensitization step platelets are washed and solubilized in a non-ionic detergent. Centrifugation, supernatant lysate is added to wells of microplate containing immobilized goat-anti mouse immunoglobulin, and is used to then detect the immune complexes with an enzyme labeled anti-human globulin. After washing, substrate and stopping solution are added in turn. OD is measured and compared with the cut off in a manner similar to the ELISA technique.

Because MAIPA is a highly sensitive technique, several laboratories considered it as a reference technique for HPA antibody detection. The MAIPA is usually used to detect HPA antibodies in Europe, although in some laboratories sera are first screened by flow cytometry, MAIPA is only used for antibody identified after positive in the screen. However, MAIPA is time-consuming, high cost and complicated technique which is not easily available and it is

difficult to perform in routine use. It may be used in larger institute or reference laboratories. Schallmoser et al. (2006) reported that existing of HPA-3 system antibodies may be difficult to detect by MAIPA and this finding was in agree with Harrison et al (2003). They demonstrate that antibodies to HPA-3 detected only in whole platelet assay (Schallmoser et al., 2006 and Harrison et al., 2003).

B.5 Standard microlymphocytotoxicity test (LCT)

HLA class I antigens present mainly intrinsic determinants of platelets, and soluble HLA antigens can be taken up by human platelets from plasma in small amounts (Santoso, 1986). Antibodies react with HLA class I antigens on platelet-cell surfaces is associated with platelet refractoriness. Platelet refractoriness in thrombocytopenic patients is most often associated with immunization to HLA class I antigens. Terasaki and McClelland (1964) had developed the microlymphocytotoxicity test. Lymphocytes are used because they express both class I and class II molecules.

The principles of a LCT are described as follows. Lymphocytes are incubated with serum in the presence of complement, and membrane-attached complexes induced pores on lymphocyte cell membranes. After staining with eosin, the cells that have sustained membrane damage will take up the dye and appear as dead cells microscopically. Undamaged cells will not take up the dye and will appear refractile and unstained under the microscope.

LCT has been applied to evaluate the influence of HLA antibodies on platelet transfusion. The standard LCT was also the system most frequently applied in detecting HLA antibodies by the participating laboratories of the Platelet Workshop 2000 (Kurz et al., 2001). Brand showed that 34% (125/367) of sera reactive in the ELISA were found to be negative when using LCT technique (International Forum, 2003). Levin and his colleagues showed that ELISA was more frequently positive (19/185) than the LCT, showing its superior sensitivity toward detecting alloimmunization (Levin et al., 2003). Kurz et al. demonstrated the superiority of the MAIPA to the LCT in detecting HLA antibodies. In 12 of 22 patients, HLA antibodies were detected by MAIPA but not by the LCT technique (Kurz et al., 2001). The MAIPA test, using a monoclonal antibody directed against the β2-microglobulin of HLA class I molecules, appears to

be a sensitive and specific test. These indicated that LCT test was not a sensitive technique for the detection of HLA antibodies when compared with ELISA and MAIPA.

In Thailand, LCT is commonly used for HLA antibody screening and crossmatching for both purposes, platelet antibody screening and organ transplantation. The LCT was chosen because of the small amount of sera required to perform the test, with consequence low costs. It was also easy to perform allowing the testing of a large number of samples. The drawbacks of LCT were its insensitivity, time-consuming and subjective results.

According to recent studies in many laboratories in Europe, America and Japan several techniques, such as MAIPA, LCT, SPRCA and flow cytometry technique have been implemented for the screening of platelet-reactive antibodies (International Forum, 2003). Although MAIPA is highly sensitive and very specific there are some limitations when applying it in routine work. It is complicated to perform, costly, time consuming and special instruments are needed. Other techniques such as LCT and SPRCA have long been used in our laboratory and others in Thailand. LCT is a technique that needs skilled personnel and the resulting measurement is subjective. SPRCA is quite insensitive and the result is also subjective. Flow cytometry is an interesting technique with high sensitivity and is less subjective for platelet-reactive antibody detection. So our interest is to evaluate these three methods in order to implement the proper methods suitable for routine investigations in our laboratory with cost-effectiveness.

Objective

Our objective is to compare the sensitivity and specificity of three techniques commonly used in Thailand to detect platelet-reactive antibodies which are standard microlymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometry technique. Cost-effectiveness analysis and the amount of time needed to perform the test will also be assessed to find out the suitable method for our routine investigation.

CHAPTER 2

RESERCH METHODOLOGY

MATERIALS

1. Chemicals and reagents

Chemicals	Molecular weight	Source
Bovine serum albumin	67,000	Sigma, USA
Calcium chloride ($CaCl_2$)	147.02	Sigma, USA
Chloroquine disulphate ($C_{18}H_{29}ClN_3.2H_3PO_4$)	515.92	Sigma, USA
Disodium hydrogen phosphate (Na_2HPO_4)	141.98	Merck, Germany
Eosin Y ($C_{20}H_6Br_4Na_2O_5$)	691.85	Sigma, USA
Ethylenediaminetetraacetic acid, EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$)	372.2	Sigma, USA
Glycine (H_2NCH_2COOH)	75.07	Merck, Germany
Paraformaldehyde ($HO(CH_2O)_nH$)	30.03	Sigma, USA
Potassium phosphate (KH_2PO_4)	136.29	Merck, Germany
Sodium bicarbonate (Na_2CO_3)	105.99	Merck, Germany
Sodium chloride (NaCl)	58.44	Sigma, USA
Sodium hydrogen carbonate ($NaHCO_3$)	84.00	Merck, Germany

1.1 Reagents for LCT

(1) Phosphate buffer saline (PBS) pH 7.2-7.4

Na_2HPO_4	0.724 g
KH_2PO_4	0.21 g
NaCl	7.65 g

All chemicals were dissolved in distilled water (DW). The solution was toped up to one liter with DW and the pH adjusted to 7.2 to 7.4.

(2) Lymphoprep; Nyegard, Norway

(3) Bovine thrombin 100 IU/ml

(4) Trypan blue

(5) Rabbit complement, One Lambda Inc, USA

(6) Five percent of Eosin Y

1.2 Reagents for SPRCA

Reagent preparation procedures were described as follow:

(1) 0.2 M Chloroquine solution pH 4. 0

Chloroquine disulphate 41 g was dissolved in 400 ml of PBS/EDTA at 40 °C. The solution was left to cool down to room temperature (25-27 °C) and the pH was adjusted to 4.0. The solution was then filtered through a 0.45 µm membrane filter and stored in the dark at room temperature.

(2) 1.9 % Glycine solution

Glycine 1.9 g was dissolved in 100 ml of DW. The working solution was then filtered through a 0.45 µm membrane filter and stored at 4 ±2 °C.

(3) 1% Paraformaldehyde (PFA)

PFA 1 g was dissolved in 100ml of PBS at 70 °C. The solution was allowed to cool down to room temperature and the pH was adjusted to 7.2 with NaOH. The working solution was then filtered through a 0.45 µm membrane filter and stored at 4 ±2 °C.

(4) Coating buffer

Na_2CO_3	0.795 g
$NaHCO_3$	1.465 g
DW	500 ml

After adjusted the pH to 9.6, the solution was filtered through 0.45µm membrane filter and stored at 4 ±2 °C.

(5) IgG-sensitized red cell (indicator cell)

The preparation procedures were described in the method session.

(6) 0.2 % BSA in NSS

(7) 0.2 % BSA in PBS

(8) Rabbit anti-human thrombocyte, DakoCytomation, Denmark

(9) Anti-human globulin (polyspecific), Thai national blood centre

1.3 Reagents for flow cytometry

(1) PBS/EDTA

Na_2HPO_4	2.743 g
Na_2EDTA	0.21 g
NaCl	7.65 g

All chemicals were dissolved in DW. The solution was topped up to one liter with DW, and then the pH was adjusted to 7.2 to 7.4. The working solution was filtered through a 0.45 μm membrane filter and stored at 4 ±2 °C.

(2) New born calf serum, Sigma, USA.

(3) Fluorescein isothiocyanate (FITC) conjugated F(ab)2 goat-antihuman IgG, Immunotech, France.

(4) One percent paraformaldehyde, Sigma, USA

(5) Sheath fluid (Isoton) Immunotech, Beckman Coulter, France

(6) Cleaning agent, Coulter Clenze, Beckman Coulter Inc, Canada

(7) Flow check, Fluorospheres, Beckman Coulter Inc, Ireland

(8) Flow set, Fluorospheres, Beckman Coulter Inc, Ireland

(9) Phycoerythrin (PE) conjugated anti-CD41, Immunotech, Beckman Coulter, France

(10) Isotype control (IgG1-FITC–IgG1-PE–IgG1-PC5), Immunotech), Beckman Coulter, France

2. Instruments and Equipments

Blood bank freezer (-70°C), Forma Scientific Inc, USA

Clay Adam Serofuge, Beckton Dickinson, USA

Cytomics FC500 flow cytometer, Beckman Coulter, USA

Inverted phase contrast microscope, Olympus, USA

KOKUSAN CH-103 microplate centrifuge, Japan

Light microscope, Olympus, USA

Microfuge E, Beckman, USA

Microsyringe with repeating dispenser, Hamilton, USA

MSE centrifuge (for conical tube), UK

Multichanel syringe, Nichiryo co.ltd., Japan

pH-meter, Orion, USA

Vortex-mixer, vortex genie 2, Scientific Industries, USA

Water bath, Memmert, Germany

3. Miscellaneous

Autopipettes, Gilson, France

Beaker, Pyrex, USA

Conical tube, BD Falcon, USA

Capillary pipette, Pyrex, USA

Filter paper No.2, Whatman, UK

13 x 75 mm FACS tube, BD Falcon, USA

Hemacytometer, Fisher Scientific Inc, USA

Magnetic stirrer, USA

Microcentrifuge tube (Fisher tube), Fisher Scientific Inc, USA

Pipette tips, BD Falcon, USA

Round button microtiter plate, NUNC corp., Denmark

Terasaki tray, Robbins Scientific, USA

Serological pipette, Pyrex, USA

Membrane filter (pore size 0.45 μm), UK

Volumatric flask, Pyrex, USA

Magnetic stirrer, USA

Filter paper No.2, Whatman, UK

4. Subjects and samples

4.1 Subjects

The study was carried out from January, 2005 to October, 2008. The study was approved by the ethics committee of the Faculty of medicine, Prince of Songkla University. Fifty-three subjects were recruited. Ten healthy blood donors were studied as control subjects. Forty-three patients were classified as studied subjects. The subjects' criteria were described as followed.

4.1.1 Ten healthy blood donors who had neither a history of blood transfusion nor a pregnancy were recruited as a control study group.

4.1.2 Patients who had platelet refractoriness and requested for platelet antibody investigation.

4.1.3 Idiopathic thrombocytopenic purpura (ITP) patients.

4.1.4 Mothers of the neonates who had thrombocytopenia and requested for platelet antibody investigation.

4.2 Samples

4.2.1 Studied samples

4.2.1.1 From control subjects 3-5 ml of clotted blood was collected. Blood samples were centrifuged and sera were separate and stored at -70^{o}C until used.

4.2.1.2 Five milliliters of blood were collected from forty-three patients and sent to the immunology laboratory, Blood Bank and Transfusion Medicine Unit of Songklanagarind Hospital, Songkhla. Sera were prepared as in the control subjects above.

4.2.2 Positive and negative control sera preparation

4.2.2.1 A positive control for anti-HPA-1a antibody was kindly provided by the HLA laboratory of the Thai National Blood Centre.

4.2.2.2 A positive control for anti-HLA class I antibody was collected from multi-transfused patients at Songklanagarind Hospital.

4.2.2.3 A negative control serum was prepared from the pooled plasma of 3 male, group AB blood donors. Serum was prepared by treating 300 milliliters of pooled plasma with 7.41 grams of 24.7% $CaCl_2$ and then filtered with No.2 filter paper (Whatman, UK) to eliminate lipid and cell debris. Serum was then screened for HLA antibody and had a negative reaction in 20 lymphocyte panel cells when using the LCT technique. Complement in the control serum was inactivated by being treated at 56 $^{\circ}$C for 30 minutes with agitation every 5 minutes. Then the serum was kept at room temperature (25-27°C) and aliquoted for 1 ml and then stored at -70°C until use.

METHODS

1. Lymphocytes and platelet screening cells preparation

In the LCT technique the target cells were lymphocytes which were prepared as mononuclear cells and used for HLA class I antibody screening. In SPRCA and flow cytometry assays platelets were used as the target cell for HLA and HPA antibody screening. All target cells were prepared from six donor subjects with blood group O. Five milliliters of whole blood were drawn from the donors in acid citrate dextrose (ACD), and target cells were freshly prepared. Target cells were not typed for HLA class I or HPA antigens because six different genotypic donors may have various antigens which possibly cover all common HLA and HPA antigens. The target cell preparation was performed as follows.

1.1 Mononuclear cell preparation for LCT

(1) Warmed lymphocyte separation medium (lymphoprep, Norway) to room temperature (25-27 $^{\circ}$C) before using the solution.

(2) Mixed 5 milliliters of whole blood with 2.5 ml of PBS in a 15 ml conical tube then underlayered diluted blood with 2.5 ml of lymphoprep.

(3) Centrifuged the sample at 1,000 g at room temperature for 15 minutes.

(4) The mononuclear cells interface was gently transferred by Pasteur pipette to a 15 ml conical tube and then washed once with PBS by centrifugation at 1,000 g for 10 minutes.

(5) The pellet was resuspended in a microcentrifuge tube with PBS, and platelet elimination was done by precipitating with 50 microliter of bovine thrombin (concentration 100 IU/ml).

(6) The sample tube was briefly centrifuged, and then cell supernatant was transfered to another microcentrifuge tube and washed once with PBS by centrifugation at 700 g for 1.5 minutes.

(7) The supernatant was discarded and the cell pellet was resuspended with PBS.

(8) For viability testing and adjustment of cell density 10 µl of cell suspension was mixed with 10 µl of 0.2% trypan blue in a 1.5ml microcentrifuge tube. The viability of the cells was estimated and the cell density was adjusted to 1 X 10^6cells/ml. The accepted viability of cells was more than 85 %.

1.2 Platelet suspension preparation for SPRCA

1.2.1 Preparation of untreated platelets

(1) Whole blood was centrifuged at 500g for 10 minutes in bench-top and platelet-rich plasma (PRP) was obtained.

(2) The PRP was transferred to a 13X75 mm plastic tube and froze at -70 °C for 1 hour or more. Then the frozen PRP was thawed at 37° C and washed twice with 0.2% BSA in 0.9%NaCl solution by centrifugation at 1,000g for 4 minutes. The supernatant was discarded and the platelet pellet was resuspended in 1 ml of 1% paraformaldehyde, then the mixture was incubated for 5 minutes at room temperature (25-27 °C).

(3) The mixture was washed twice with 0.2% BSA in 0.9%NaCl solution by centrifugation at 1,000g for 4 minutes. The supernatant was discarded and the platelet pellet was resuspended in 0.2% BSA in 0.9%NaCl solution adjusted to approximately 1X10^8/ml and used as untreated platelet target cells.

1.2.2 Preparation of chloroquine-treated platelets

HLA antigens on platelet surfaces were removed by treating platelets with chloroquine. HPA antigens still remained on platelet surfaces.

(1) Two milliliters of 0.2 M solution of chloroquine disulphate with a pH of 4.0 was added to the 100 µl of untreated-platelet suspension from 1.2.1 (3).

(2) The platelet and chloroquine mixture was incubated for 30 minutes at room temperature (25-27 ^{o}C). The mixture was then washed 3 times with 0.2% BSA in 0.9%NaCl solution by centrifugation at 1,000g for 4 minutes.

(3) The supernatant was discarded and the platelet pellet was resuspended in 1 ml of 1% paraformaldehyde, then the mixture was incubated for 5 minutes at room temperature (25-27 ^{o}C). The mixture was washed twice with 0.2% BSA in 0.9%NaCl solution by centrifugation at 1,000g for 4 minutes.

(4) In the final wash the supernatant was discarded and the platelet pellet was resuspended in 0.2% BSA in 0.9%NaCl solution and adjusted to approximately 1X10^{8}/ml and used as chloroquine-treated platelet target cells.

1.3 Platelet suspension preparation for Flow cytometry

1.3.1 Preparation of untreated platelet

(1) Whole blood was centrifuged at 500g for 10 minutes in bench-top and platelet-rich plasma (PRP) was obtained.

(2) The PRP was transferred to a 13X75 mm. plastic tube and washed twice with 0.2% BSA in PBS/EDTA solution by centrifugation at 1,000g for 4 minutes.

(3) The supernatant was discarded and the platelet pellet was resuspended in 0.2% BSA in PBS/EDTA and used as untreated-platelet target cells.

1.3.2 Preparation of chloroquine-treated platelets

(1) Two milliliters of 0.2 M solution of chloroquine disulphate with a pH of 4.0 was added to the100 µl of untreated-platelet suspension from 1.3.1 (3).

(2) The platelet and chloroquine mixture was incubated for 30 minutes at room temperature (25-27 $^{\circ}$C). Then the mixture was washed 3 times with 0.2% BSA in PBS/EDTA by centrifugation at 1,000g for 4 minutes.

(3) In the final wash the supernatant was discarded and the platelet suspension was resuspended in 0.2% BSA/EDTA and used as chloroquine-treated platelet target cells.

(4) One hundred microlitters of Chloroquine-treated platelets and untreated platelets were incubated with 1 ml of new born calf serum at room temperature for 20 minutes.

(5) The platelet suspensions were washed 3 times with 0.2% BSA in PBS/EDTA by centrifugation at 1,000g for 4 minutes.

(6) The platelet button was resuspended in 0.2% BSA/PBS/EDTA and the concentration was adjusted to approximately 1 X 10^8 /ml, then stored at 4-8 °C for up to 7 days before use.

1.4 Preparation of IgG-sensitized red cells (indicator cells)

IgG-sensitized red cells were used as indicator cells for reading the reaction of SPRCA. When the patient has IgG antibodies (either HLA or HPA antibodies) the antibodies will attach to the platelet screening cells. The IgG (anti-D) sensitized red cells, which are used as indicator cells, will form a complex with antihuman globulin antibody (anti-IgG). When the tests have positive antibody reactions, the sera antibodies will bind to the coated platelets on the plate. The added antihuman globulin will also bind to the sera antibodies and the added indicator cells will indicate the positive sera antibodies by binding to antihuman globulin antibodies which will appear as dispersed cells on the bottom of the tested wells. Conversely, the tested sera did not have the positive antibodies and, thus, resulted in a negative reaction, since the antihuman globulin antibodies will not adhere to the unbound-antibody platelets. When indicator cells were added and centrifuged all the indicator cells will not be dispersed on the well bottom and will appear as a smaller red cell button (figure8). The IgG-sensitized indicator cells were prepared as follows.

(1) ACD blood was prepared from three blood donors which were O Rh positive. Then packed red blood cells were pooled and washed 3 times with 0.9% NaCl solution by centrifugation at 1,000g for 4 minutes.

(2) Pellet red blood cells were resuspended and the concentration was adjusted to 5% cell suspension in 0.9%NaCl solution.

(3) The red cells were sensitized with anti-D (IgG) antibodies by adding one milliliter of 5% cell suspension with anti-D (titer 1:100). This was then incubated at 37 °C for 30 minutes.

(4) Cells were washed 3 times with 0.9%NaCl solution and the stock concentration was adjusted to 2.5 % cell suspension. The optimal indicator cell was adjusted by using the direct antiglobulin test. The grading positive reaction 1+ was the optimum.

(5) Indicator cells were stored at 4 ±2 °C for up to 1 month.

2. Experimental procedures

Study sera were tested in parallel using the three methods which were standard microlymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometry. Negative and positive controls were applied in every test and considered valid if positive and negative controls gave the proper reactions.

2.1 Standard microlymphocytotoxicity test (LCT)

LCT was performed according to Levin et al. (2003) using the method originally described by Terasaki et al. (1964). Detailed experimental procedures were described.

(1) One microliter of patient sera, including negative control and positive control, were incubated with 1 µl of cell suspension in a Terasaki tray at room temperature for 30 minutes.

(2) Rabbit complement 5 µl were added to each separate well.

(3) After incubating at room temperature for 60 minutes, 5 µl of 5% eosin-Y was added for 2 minutes, after which 10 µl of formaldehyde was added.

(4) The tray was covered and left for at least 4 hours at room temperature.

(5) Dead cells were read by inverted phase contrast microscopy and reactions were graded

using a grading system which corresponds to the percentage of dead cells within each well.

0 = Not readable

1 = 0-10% dead cells

2 = 11-20% dead cells

4 = 21-50% dead cells

6 = 51-80% dead cells

8 = 81-100% dead cells

(6) The reaction against the screening cells was considered to be positive when dead cells were more than 50%. The examples of positive and negative reactions are shown in figure 8.

(7) If two or more of 6 donors showed a positive reaction, the patient was considered immunized against a broad range of HLA antibodies according to the recent study (Levin et al., 2003).

2.2 Solid phase red cell adherence assay (SPRCA)

SPRCA was performed according to the protocol from the National Blood Centre, Thai Red Cross Society and Lown, 1991 using the method originally described by Shibata et al., 1981. Detailed experimental procedures were described.

(1) Fifty microlitters of 20 μg/ml concentration of rabbit antihuman thrombocyte in pH 10 carbonate buffer was added to a round bottomed microplate.

(2) The microplate was incubated for 2 hours at 22 °C before use.

(3) Platelet monolayers were prepared by discarding the buffer from the microplate, rinsing the wells with 0.2%BSA/PBS 3 times and adding 50 μl of platelet suspension containing both chloroquine-treated and untreated platelets to the required number of microplate wells.

(4) The microplate was centrifuged at 1,700 rpm for 2 minutes using a microplate centrifuge (KOKUSAN, Japan).

(5) The supernatant was discarded and the microplate was incubated at 37 °C for 15 minutes.

(6) The microplate was washed off with 0.2% BSA/PBS 6 times to remove non-adherent platelets.

(7) One hundred microliters of a 1.9% glycine solution was added to platelet monolayers in the microplate wells.

(8) Fifty microliters of test sera, negative control, positive control for HLA antibodies and positive control for HPA antibodies were added to the platelet-coated wells and incubated at 37 °C for 15 minutes.

(9) The microplate was washed off with 0.2% BSA/PBS 6 times, and unbound-form immunoglobulins were removed.

(10) Fifty microliters of 0.4% suspension of blood group O Rh(D) positive red cells, sensitized with anti-D (indicator cells), together with 50 μl of anti-human IgG (dilution 1:4) were added.

(11) The microplate was gently mixed and centrifuged at 2,700 rpm for 2 minutes using a microplate centrifuge (KOKUSAN, Japan).

(12) The reactions were read by examining each for either effacement (a reactive or positive reaction result) or a button of agglutinated red cells (a non-reactive or negative reaction result). The examples of positive and negative reactions are shown in figure 8.

(13) When 2 or more out of 6 donors showed positive reactions, the patient was considered immunized against HLA class I or HPA antigens (Freedman and Hornstein, 1991).

2.3 Flow cytometry

An indirect immunofluorescence test by flow cytometry was performed with some modifications according to the protocol from the National Institute for Biological Standards and Control (NIBSC), of UK using the method originally described by Kiefel et al. (1987) and Kohler et al. (1995). Detailed experimental procedures were as followed.

2.3.1 Immunofluorescence staining

(1) For each sample to be tested 50 μl of platelet suspension (Chloroquine-treated and untreated platelets) was put into FACS tubes (Falcon 2052, USA).

(2) Fifty microlitters of test serum, including negative controls, positive controls for HLAclass I antibodies and positive controls for HPA antibodies were added into the tubes and

gently mixed.

(3) After incubation at 37 °C for 30 minutes, 4 ml of 0.2% BSA/PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(4) Supernatant was discarded and cell pellets were resuspended in 0.2% BSA/PBS/EDTA, then each tube step (3) and (4) was repeated twice.

(5) Platelet suspensions were incubated with 50 µl of FITC-conjugated $F(ab)_2$ goat-antihuman IgG (dilution 1:50) and 5 µl of PE-conjugated anti-CD41 (undiluted) at room temperature for 30 minutes in the dark, making sure the cells were fully resuspended. In order to prevent non-specific binding of the immunoglobulin via Fc receptors on the target platelets, F(ab)'2 fragment of goat antihuman IgG was used to reduce interference from Fc receptor binding (Lina et al., 2000).

(6) Four milliliters of 0.2% BSA/ PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(7) The supernatant of each tube was discarded and cell pellets were resuspended in 0.5 ml of 0.2% BSA in PBS/EDTA, then stored in the dark at room temperature (25-27°C) until flow cytometric nalysis.

2.3.2 Flow cytometric analysis

Stained cells were analyzed by flow cytometry (Cytomics FC500, Beckman Coulter, USA). This system is equipped with an argon ion laser emitting at 488 nm. A 525 nm band-pass filter was located in front of the fluorescence detector. Dot-plot histograms were generated for each sample relating the forward (FS) and side scatter (SS) signals. List mode data were acquired (CXP Software Version 2.1, 2005, Miami, FL, USA).

(1) The FS and SS amplifier gains were set to logarithmic mode for data collection. The sample flow rate should be low or medium.

(2) Debris was excluded by increasing the threshold of forward scatter signals.

(3) The fluorescence channels (FL1 and FL2 for FITC and PE conjugate, respectively) were set as logarithmic gain.

(4) Around the population of CD41 positive thrombocytes a region A was set.

(5) Approximately 10,000 cells per test sample were counted. Relative fluorescence intensity was expressed as a mean channel number (MCN).

(6) The normal range for MCN was established by using sera from 10 normal blood donors (negative control). All sera were tested against 6 platelets; the mean and standard deviation (SD) were calculated. The normal range for MCN was 25 % (mean + 3SD).

(7) MCN greater than 25% was considered positive for platelet-reactive antibodies (Worfolk and MacPherson, 1990). Therefore, positive reactions were defined when a MCN greater than 25% was recorded. The examples of positive and negative reactions are shown in figure 8.

(8) When 2 or more of 6 donors showed a positive reaction, the patient was considered immunised against HLA class I or HPA antigens (Freedman and Hornstein, 1991).

2.4 Confirmation of HLA antibody detection using mononuclear cells as the target cells

Discrepancies in the results of samples tested with flow cytometry and LCT were confirmed. Lymphocytes were used as target cells to detect HLA antibodies. The technique was described as follows.

(1) For each sample to be tested aliquot 50 µl of mononuclear cell suspension were put into FACS tubes (Falcon 2052, USA).

(2) Fifty microlitters of test serum including negative controls, positive control for HLA class I antibodies and positive control for HPA antibodies were added into the tubes and gently mixed.

(3) After incubation at 37 $^{\circ}$C for 30 minutes, 4 ml of 0.2% BSA/PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(4) The supernatant was discarded and cell pellets were resuspended in 0.2% BSA/PBS/EDTA, then each tube step (3) and (4) were repeated twice.

(5) Mononuclear cell suspensions were incubated with 50 µl of FITC-conjugated $F(ab)_2$ goat-antihuman IgG (dilution 1:50) at room temperature for 30 minutes in the dark, making sure cells were fully resuspended.

(6) Four milliliters of 0.2% BSA/ PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(7) The supernatant in each tube was discarded and cell pellets were resuspended in 0.5 ml of 0.2% BSA in PBS/EDTA, then stored in dark at room temperature (25-27^{o}C) until flow cytometric analysis.

(8) Stained cells were analyzed by flow cytometry (Cytomics FC500, Beckman Coulter, USA). FS and SS amplifier gains were set to logarithmic mode for data collection. The sample flow rate should be low or medium. Debris was excluded by increasing the threshold of forward scatter signals.

(9) The fluorescence channels (FL1 and FL2 for FITC and PE conjugate, respectively) were set as logarithmic gain.

(10) A region A was set around the lymphocyte population.

(11) Approximately 10,000 cells per a test sample were counted. The relative fluorescence intensity was expressed as a mean channel number (MCN).

(12) MCN greater than 25% was considered positive for platelet-reactive antibodies (Worfolk and MacPherson, 1990). The examples of positive and negative reactions are shown in figure 9.

(13) When 2 or more of 6 donors showed a positive reaction, the patient was considered immunized against HLA class I or HPA antigens (Levin et al, 2003).

2.5 Identifying HPA antibody from HLA antibody using adsorbed serum against a panel of screening platelets.

To classify type of antibodies whether they were mixture of HLA and HPA antibodies or single HPA antibody, HPA absorption technique was applied using chloroquine-treated platelets as an adsorbent cell. Then adsorbed serum was tested with untreated platelets and chloroquine-treated platelets using the flow cytometry technique. The technique was described as follow.

(1) One milliliter of patient serum was incubated with 0.5 ml of chloroquine-treated platelets from 1.3.2 (6) at 37^{o}C for 1 hour.

(2) After centrifugation at 1,000g for 4 minutes, the supernatant was collected and was used as adsorbed serum.

(3) For each sample to be tested aliquot 50 µl of platelet suspension (Chloroquine-treated and untreated platelets) was put into FACS tubes (Falcon 2052, USA).

(4) Fifty microlitters of adsorbed serum including negative controls, positive controls for HLAclass I antibodies and positive controls for HPA antibodies were added into the tubes and gently mixed.

(5) After incubation at 37 $^{\circ}$C for 30 minutes, 4 ml of 0.2% BSA/PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(6) The supernatant was discarded and cell pellets were resuspended in 0.2% BSA/PBS/EDTA. Then each tube step (3) and (4) were repeated twice.

(7) Platelet suspensions were incubated with 50 µl of FITC-conjugated $F(ab)_2$ goat-antihuman IgG (dilution 1:50) and 5 µl of PE-conjugated anti-CD41 (undiluted) at room temperature for 30 minutes in the dark, making sure cells were fully resuspended.

(8) Four milliliters of 0.2% BSA/ PBS/EDTA wash buffer was added and centrifuged at 1,000g for 4 minutes.

(9) The supernatant each tube was discarded and cell pellets were resuspended in 0.5 ml of 0.2% BSA in PBS/EDTA, then stored in the dark at room temperature (25-27°C) until flow cytometric analysis.

(10) Flow cytometry analysis was performed using the same conditions as described in 2.3.2 (1) to (8). The examples of positive result and negative result reactions were showed in figure 10.

3. Data analysis

The sensitivity and specificity of each technique was compared with a reference assay. The following definitions of sensitivity and specificity were used.

$$\text{- Sensitivity} = \frac{\text{true positive sera}}{\text{true positive + false negative sera}} \times 100$$

$$\text{- Specificity} = \frac{\text{true negative sera}}{\text{true negative + false positive sera}} \times 100$$

	Reference method		
Test method		Positive	Negative
	Positive	True positive	False positive
	Negative	False negative	True negative

4. Statistical analysis

For every combination of two techniques, a 2 X 2 table was generated, and Pearson's chi-square test was calculated to test for independence. The p values less than or equal to 0.05 were considered as correlated significantly. For the strength of agreement, the Kappa coefficient (κ) was determined. All values of statistics were calculated with the SPSS version 16.0 software program.

4.1 Chi-square test

A 2x2 table was first drawn and actual numbers of occurrences placed there in. Then the expected frequencies in each cell were calculated. The expected frequency in the cell was a product of the relevant row and column totals divided by the sample size (grand total; N = a+b+c+d). For the cell with an observed frequency 'a', for example, the expected value was (a+b)(a+c)/N. The difference between observed (O) and expected (E) values (residual) was calculated. The χ^2 was calculated as the sum of $(O-E)^2/E$ for all four cells: $\chi^2 = [\sum (O-E)^2/E]$. The general layout of a contingency table for a test correlation study is as follows.

A 2x2 table		Reference method		Row totals
		Positive	Negative	
Test method	Positive	a	b	a+b
	Negative	c	d	c+d
Column totals		a+c	b+d	N=a+b+c+d

4.2 Kappa coefficient of agreement

The formula for calculating Kappa values is as follows. Kappa = (Observed agreement - Chance agreement)/(1 - Chance agreement). Kappa's possible values were constrained to the interval (0-1); $\kappa = 0$ means that agreement is not different from chance, and $\kappa=1$ means perfect agreement.

Kappa	Strength of agreement
0.00	Poor
0.01-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.00	Perfect agreement

Figure 8. Examples of negative and positive results using LCT (A), SPRCA (B) and the flow cytometry technique (C1-C3).

Figure 8-A. Microlymphocytotoxicity test (LCT)

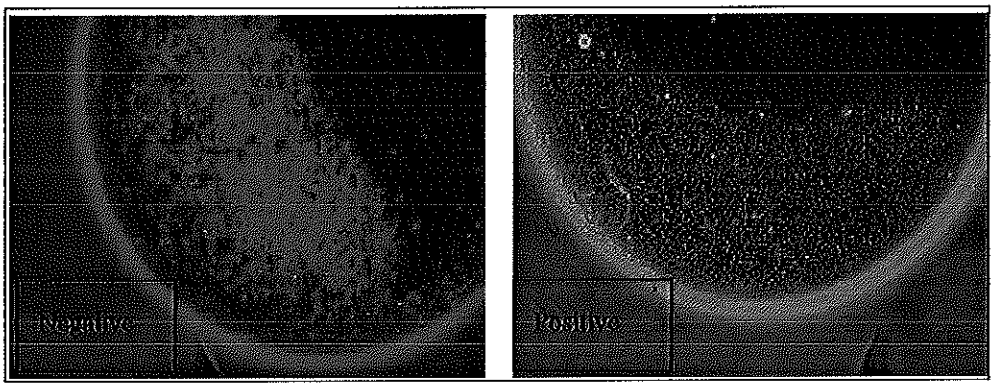


Figure 8-B. Solid phase red cell adherence assay (SPRCA)

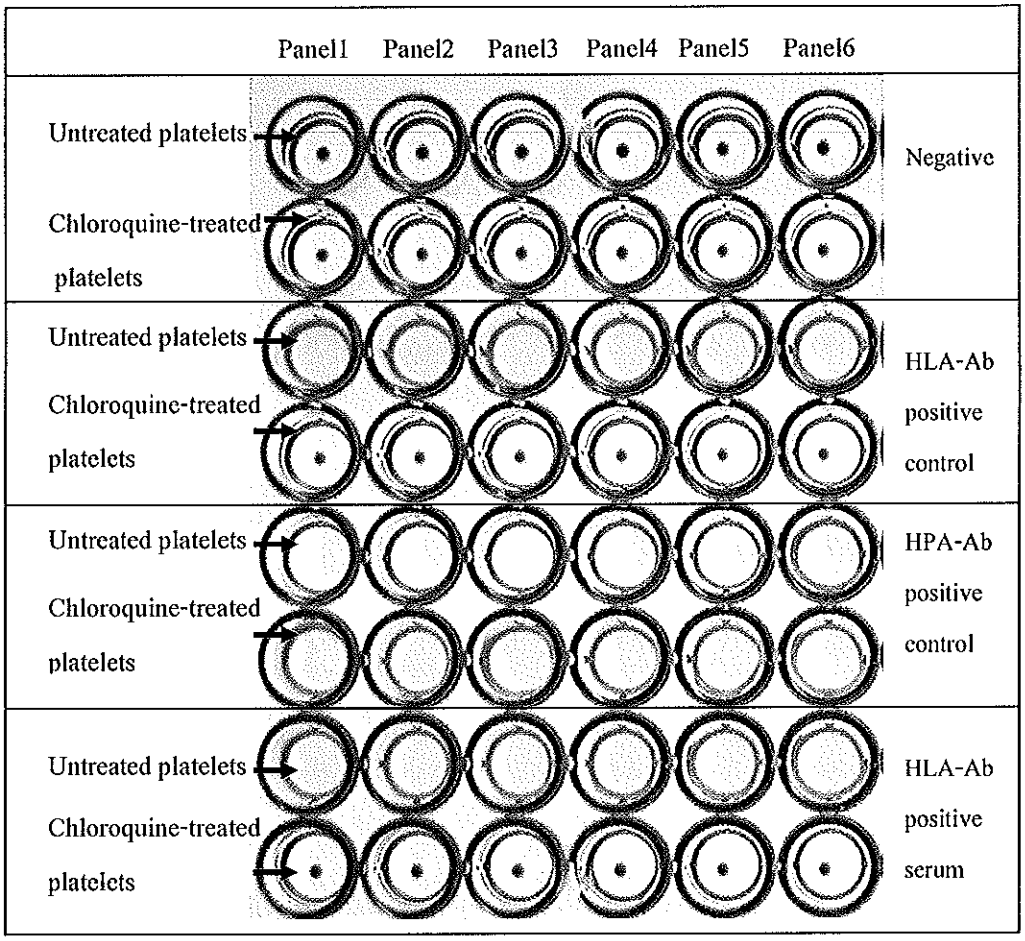
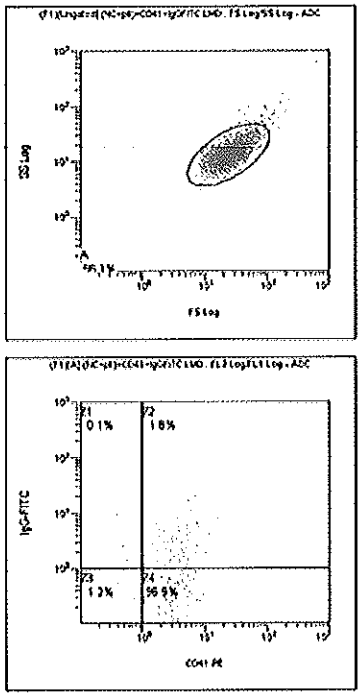
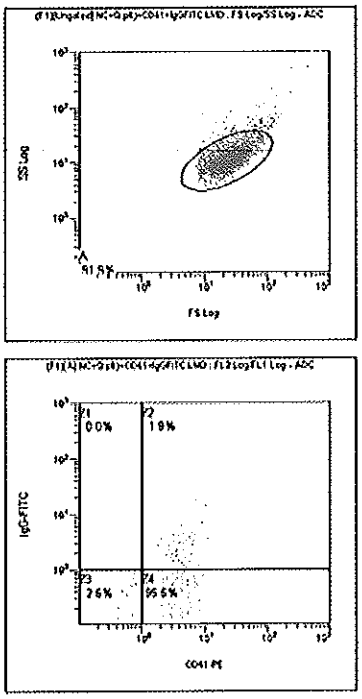


Figure 8-C1. Flow cytometry

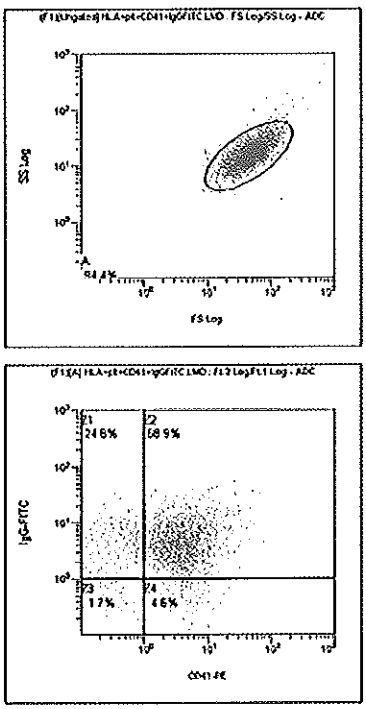


Negative control+ untreated platelets



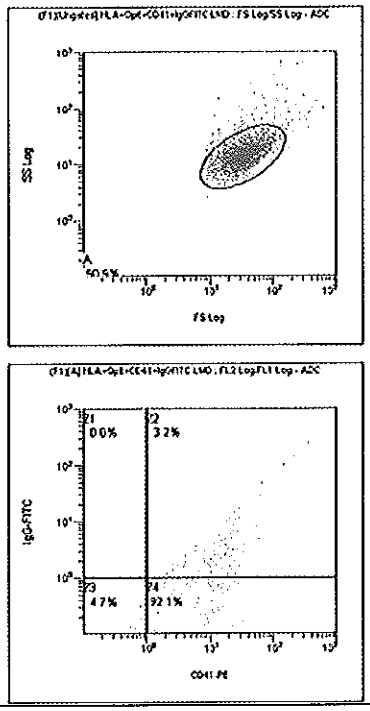
Negative control+ chloroquine-treated platelets

Figure 8-C2.



Positive control (HLA antibodies)

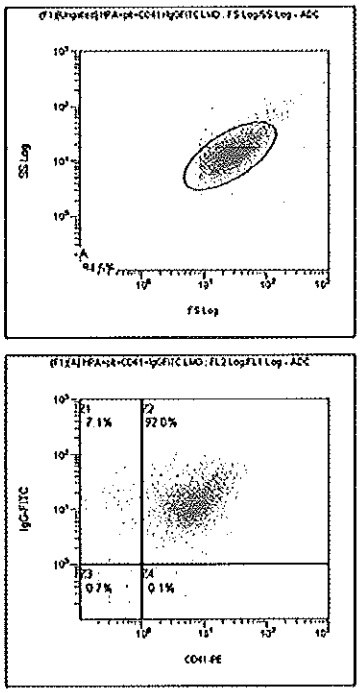
+ untreated platelets



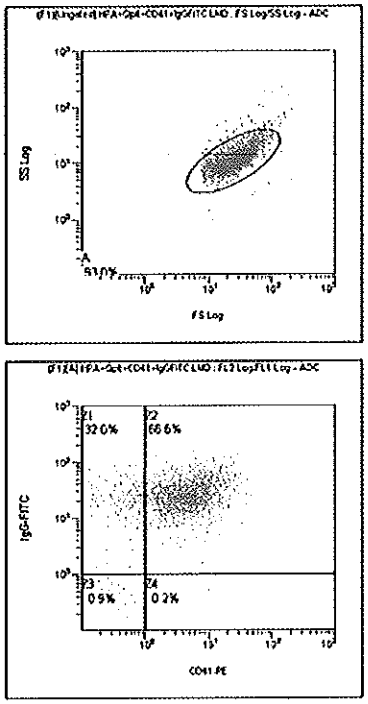
Positive control (HLA antibodies)

+ Chloroquine-treated platelets

Figure 8-C3.



Positive (HPA antibodies)
+ untreated platelets



Positive (HPA antibodies)
+ Chloroquine-treated platelets

CHAPTER 3

RESULTS

1. Results of the testing samples

Platelet antibodies were studied in sera of 10 healthy blood donors who were used as a negative control group. All subjects were tested by LCT, SPRCA and flow cytometry. The results showed negative platelet reactive antibodies either HLA or HPA antibodies in all 10 sera (table 6).

In the studied sera from 43 patients which were tested with LCT, SPRCA and flow cytometry techniques. The subject characteristics were 32 female and 11 male with mean age was 36.12 years old (range 8-75 years). Twenty-one samples were from hemato-oncology patients who were diagnosed with leukemia or lymphoma and had developed platelet refractoriness during the course of chemotherapy. Thirteen samples were from patients who had been diagnosed with ITP. Five samples were from the mothers whose babies suspected NAIT. Four samples were from other thrombocytopenic patients whose diagnoses were aplastic anemia, MDS, myelofibrosis, coagulopathic bleeding with platelet refractoriness (table7). Each serum was screened for both HLA reactive antibodies by LCT, SPRCA and flow cytometry and HPA reactive antibodies by SPRCA and flow cytometry.

The primary results found platelet reactive antibodies in 18 out of 43 sera using LCT assay and found 19 out of 43 sera when using SPRCA and found 23 out of 43 sera when using flow cytometry technique. When considering the number of positive screening cells from the screen panel found that the LCT technique gave totally reactive antibodies to 76 cells out of 258 screening cells. SPRCA gave totally reactive 95 cells out of 258 screening cells. Flow cytometry gave total reactive to 110 cells out of screening 258 cells. There were 8 discrepancy sera in platelet reactive antibodies detection when compare between three methods (table7). Eight discrepancy sera (patient 23, 26, 37, 39, 40, 41, 42 and 43) were associated with HLA antibody detections and 4 discrepancy sera (patient 40, 41, 42 and 43) were associated with HPA antibody findings.

Results of HLA antibody tests study

From eight discrepancy sera (patient 23, 26, 37, 39, 40, 41, 42 and 43), patient 23 and 37 gave HLA antibody reactive to some screening cells with LCT (3 out of 6 cells) and untreated platelets (3 and 4 out of 6 platelet panels) by flow cytometry technique considering HLA reactive antibodies were weak while SPRCA showed negative reaction. In serum of patient 26 the reactive antibody was found weakly positive in SPRCA while LCT and flow cytometry showed negative reaction. In patient 39 flow cytometry gave HLA reactive antibody (average of mean fluorescence intensity was 73%) against untreated platelet panels but gave negative reactions against chloroquine-treated platelets, considering sole HLA antibody was presented. This serum was considered true positive reactions because LCT and SPRCA tests of this serum were repeated and repeated results were negative. Repeat flow cytometry technique was also performed in patient 39 using a panel of 6 mononuclear cells or lymphocytes as target cells to determine HLA antibodies against HLA antigens on lymphocytes. This study showed the present HLA antibody in the serum as showed in figure 9. These can be indicated that serum from patient 39 contained HLA antibody and gave false negative results by LCT and SPRCA. In patients 40, 41, 42 and 43 the platelet reactive antibodies were found only in target platelets but non reactive against lymphocyte target cells in LCT test. Whether or not HLA antibody was presence together with HPA antibody in sera needed to be further determined as in section below.

Results of HPA antibody tests study

Four discrepancies in HPA antibody tests by three methods were found (patient 40, 41, 42 and 43). In flow cytometry studied, 4 out of 23 antibody reactive samples found the positive reactions against both untreated platelets and chloroquine treated platelets. In SPRCA there were 2 out of 23 antibody reactive samples (patient 40 and 41) that the positive reactions were found against both untreated platelets and chloroquine-treated platelets where as patients 42 and 43 had negative reactions (table 7). In these sera of patients 40, 41, 42 and 43 either mixtures of HLA and HPA antibodies or sole HPA antibody could not classified in these sera as untreated platelet comprise both HLA class I and HPA antigens. They were more likely to be sole HPA antibody

because the LCT test determined sole HLA antibodies in these sera were non reactive. The confirmation studies were performed to classify type of antibody. The confirmation tests were done (in patient 43) to classify type of antibodies whether they were mixture of HLA and HPA antibodies or single HPA antibody.

The patient 43 the mother whose baby suspected NAIT, serum was confirmed for HPA antibody presented in the serum. HPA absorption technique was applied using chloroquine-treated platelets as an absorbent cell. Then absorbed serum (supernatant) was tested with platelet panels both untreated and chloroquine-treated platelets using flow cytometry technique. The results showed negative in both of platelet panels, indicating that HPA antibody was absorbed and there was not HLA antibody presence in the serum (figure10). In sera of patients 42 and 43 tested by SPRCA the negative reaction against untreated platelet and chloroquine-treated platelet were considered false negative.

Table 6. Platelet antibody study in 10 healthy controls using LCT, SPRCA and flow cytometry techniques. All tests were negative for platelet antibodies

No.	Name	Result				
		LCT	SPRCA		Flow cytometry	
			Untreated platelets	Chloroquine-treated platelets	Untreated platelets	Chloroquine-treated platelets
S1	14622-51	Negative	Negative	Negative	Negative	Negative
S2	14623-51	Negative	Negative	Negative	Negative	Negative
S3	14633-51	Negative	Negative	Negative	Negative	Negative
S4	14635-51	Negative	Negative	Negative	Negative	Negative
S5	14636-51	Negative	Negative	Negative	Negative	Negative
S6	14638-51	Negative	Negative	Negative	Negative	Negative
S7	14632-51	Negative	Negative	Negative	Negative	Negative
S8	14637-51	Negative	Negative	Negative	Negative	Negative
S9	14638-51	Negative	Negative	Negative	Negative	Negative
S10	14640-51	Negative	Negative	Negative	Negative	Negative

Table 7. Results of platelet antibody studies in 43 patient sera

Code	Age (years)	Sex	Patient diagnosis	Result				
				LCT	SPRCA		Flow cytometry	
					Untreated platelets	Chloroquine - treated platelets	Untreated platelets	Chloroquine - treated platelets
Pt1 2302-50	71	female	AML-M4 with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt2 27426-49	16	female	AML with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt3 9524-50	70	female	CML with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt4 1154045	54	male	AML with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt5 24546-50	22	male	AML-M5 with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt6 30453-50	20	female	Aplastic anemia with platelet refractoriness	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt7 22240-50	12	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt8 29630-50	20	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt9 8607-50	27	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt10 497009	20	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt11 30045-51	29	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt12 10115-50	63	male	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt13 3242-51	8	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive

Table 7. Results of platelet antibody studies in 43 patient sera (continued)

Code	Age (years)	Sex	Patient diagnosis	Result				
				LCT	SPRCA		Flow cytometry	
					Untreated platelets	Chloroquine - treated platelets	Untreated platelets	Chloroquine - treated platelets
Pt14 24346-50	11	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt15 12262-51	53	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt16 30078-51	16	female	ITP	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt17 344-52	28	female	Mother serum of suspected NAIT baby	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt18 1510547	27	female	Mother serum of suspected NAIT baby	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive
Pt19 20175-51	22	female	Mother serum of suspected NAIT baby	Non reactive	Non reactive	Non reactive	Non reactive	Non reactive

Table7. Results of platelet antibody studies in 43 patient sera (continued)

Code	Age (years)	Sex	Patient diagnosis	Result				
				LCT	SPRCA		Flow cytometry	
					Untreated platelets	Chloroquine - treated platelets	Untreated platelets	Chloroquine - treated platelets
Pt20 18723-49	49	female	ALL-L2 with platelet refractoriness	Reactive (3 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt21 2475-50	60	Male	CML with platelet refractoriness	Reactive (3 out of 6)	Reactive (3 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt22 8727-51	41	Male	MDS with platelet refractoriness	Reactive (4 out of 6)	Reactive (4 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt23 8910-50	27	female	CML with platelet refractoriness	Weakly reactive (3 out of 6)	Non reactive	Non reactive	Weakly reactive (3 out of 6)	Non reactive
Pt24 1296-50	31	female	AML-M1 with platelet refractoriness	Reactive (6 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt25 10007-50	69	female	AML-M5 with platelet refractoriness	Reactive (3 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt26 6779-50	39	male	AML-M4 with platelet refractoriness	Non reactive	Weakly reactive (2 out of 6)	Non reactive	Non reactive	Non reactive
Pt27 6785-50	53	female	AML with platelet refractoriness	Reactive (2 out of 6)	Reactive (4 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt28 13431-50	43	female	AML-M4 with platelet refractoriness	Reactive (3 out of 6)	Reactive (4 out of 6)	Non reactive	Reactive (4 out of 6)	Non reactive
Pt29 12831-49	19	female	coagulopathy with platelet refractoriness	Reactive (6 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive

Table 7. Results of platelet antibody studies in 43 patient sera (continued)

No.	Age (years)	Sex	Patient diagnosis	Result				
				LCT	SPRCA		Flow cytometry	
					Untreated platelets	Chloroquine - treated platelets	Untreated platelets	Chloroquine - treated platelets
Pt30 934934	60	Male	NHL with platelet refractoriness	Reactive (6 out of 6)	Reactive (5 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt31 1522855	44	female	Leukemia with platelet refractoriness	Reactive (6 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt32 11563-48	19	Male	ALL-L2 with platelet refractoriness	Reactive (6 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt33 1287239	24	Male	AML with platelet refractoriness	Reactive (5 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt34 1117626	68	Male	AML with platelet refractoriness	Reactive (5 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt35 1143488	63	Male	AML with platelet refractoriness	Reactive (3 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (6 out of 6)	Non reactive
Pt36 1041917	47	female	CML with platelet refractoriness	Reactive (3 out of 6)	Reactive (3 out of 6)	Non reactive	Reactive (3 out of 6)	Non reactive
Pt37 13809-48	75	female	Myelofibrosis with platelet refractoriness	Weakly reactive (3 out of 6)	Non reactive	Non reactive	Weakly reactive (4 out of 6)	Non reactive
Pt38 1041917	30	female	AML with platelet refractoriness	Reactive (6 out of 6)	Reactive (6 out of 6)	Non reactive	Reactive (5 out of 6)	Non reactive
Pt39 20111-50	17	female	ITP	Non reactive	Non reactive	Non reactive	Reactive (5 out of 6)	Non reactive

Table 7. Results of platelet antibody studies in 43 patient sera (continued)

No.	Age (years)	Sex	Patient diagnosis	Result				
				LCT	SPRCA		Flow cytometry	
					Untreated platelets	Chloroquine -treated platelets	Untreated platelets	Chloroquine -treated platelets
Pt40 National Blood Centre	25	female	Mother serum of suspected NAIT baby	Non reactive	Reactive (6 out of 6)	Reactive (6 out of 6)	Reactive (6 out of 6)	Reactive (6 out of 6)
Pt41 15537-50	22	female	ITP	Non reactive	Reactive (6 out of 6)	Reactive (6 out of 6)	Reactive (6 out of 6)	Reactive (6 out of 6)
Pt42 26765-50	42	female	ITP	Non reactive	Non reactive	Non reactive	Reactive (6 out of 6)	Reactive (6 out of 6)
Pt43 1739-52	22	female	Mother serum of suspected NAIT baby	Non reactive	Non reactive	Non reactive	Reactive (6 out of 6)	Reactive (6 out of 6)
Total	**Reactive**			**18 sera (76 screening cells)**	**19 sera (95 screening cells)**		**23 sera (110 screening cells)**	
	Non reactive			**25 sera**	**24 sera**		**20 sera**	

Table 8. Summarized of HLA antibodies screening using lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometric assay (Flow) in 43 patients (11 male, 32 female)

History	Number of reactive sample			Number of non reactive sample		
	Flow	SPRCA	LCT	Flow	SPRCA	LCT
- Hematologic malignancy with platelet refractoriness (n=21)						
- Concordance	14	14	14	6	6	6
- Discrepancy	1	0	1			
	0	1	0			
- Idiopathic thrombocytopenic purpura (n=13)	1	0	0	12	13	13
-The mothers who delivered babies with suspected NAIT (n=5)	0	0	0	5	5	5
- Thrombocytopenic patients with platelet refractoriness (n=4) - Aplastic anemia - Myelodysplastic syndrome (MDS) - myelofibrosis - coagulopathic bleeding						
- Concordance	2	2	2	1	2	1
- Discrepancy	1	0	1			
Total	19	17	18	24	26	25

Table 9. HPA antibody screening using the flow cytometry technique and solid phase red cell adherence assay (SPRCA) in 43 patients (11 male, 32 female)

Patients	Number of reactive sample		Number of non reactive-sample	
	Flow cytometry	SPRCA	Flow cytometry	SPRCA
- Hematologic malignancy with platelet refractoriness (n=21)	0	0	21	21
- Idiopathic thrombocytopenic purpura (n=13)	2	1	11	12
- The mothers who delivered babies with suspected NAIT (n=5)	2	1	3	4
- Thrombocytopenic patients with platelet refractoriness (n=4); aplastic anemia, MDS, myelofibrosis, coagulopathic bleeding	0	0	4	4
Total	4	2	39	41

Table 10. Summarized of discrepancies in HLA and HPA antibodies screening using lymphocytotoxicity test (LCT), solid phase red cell adherence assay (SPRCA) and flow cytometric assay (Flow) and the results of confirmation studies

Initial results			Confirmation results			Patient number
Number of reactive sample			Number of positive sample			
Flow	SPRCA	LCT	Flow	SPRCA	LCT	
2	0	2	2	0	2	Patient no. 23 and 37
0	1	0	0	1	0	Patient no. 26
1	0	0	1	0	0	Patient no. 39
2	2	0	2	2	0	Patient no. 40 and 41
2	0	0	2	0	0	Patient no. 42 and 43

Figure 9 The results of flow cytometry technique using 6 mononuclear cells as target cells for HLA antibody detection in patient 39.

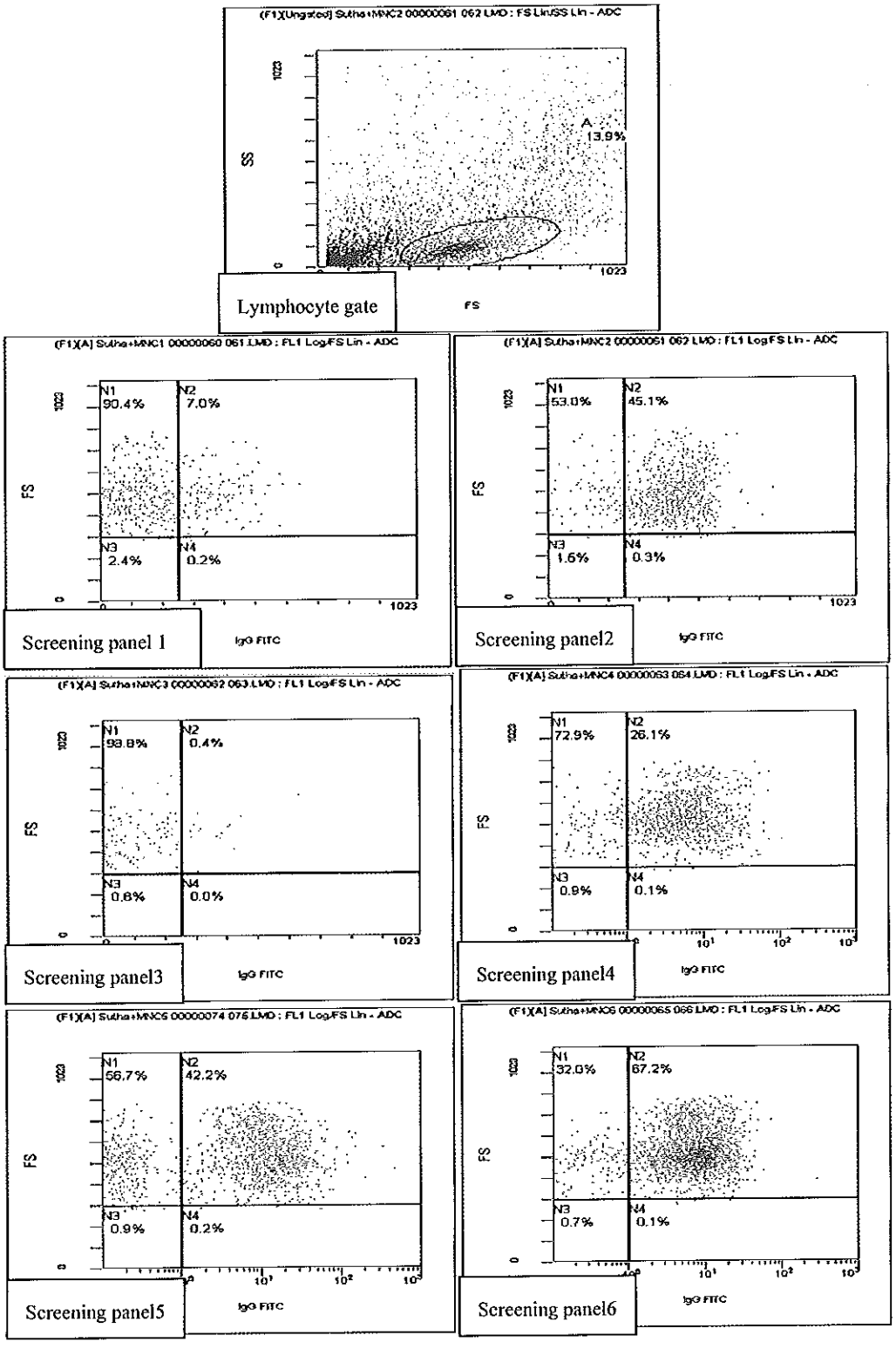
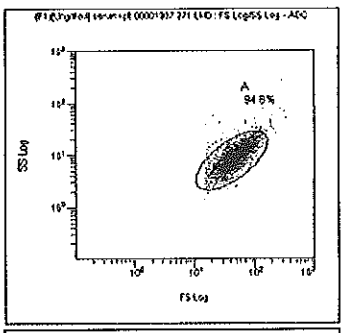
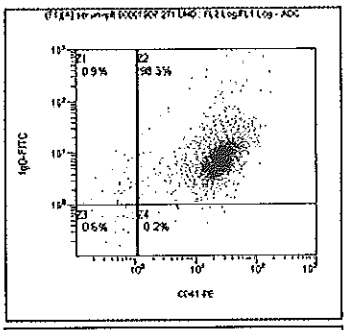


Figure 10. Result of flow cytometry in patient 43; showed positive reaction by flow cytometry technique in both chloroquine-treated and untreated platelets (A); after the serum was adsorbed by chloroquine-treated platelets, flow cytometry technique was repeated and the negative reactions were found in both of chloroquine-treated and untreated platelets (B).

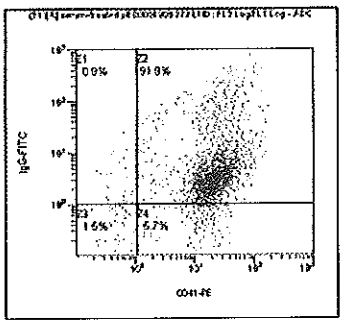
(A)



platelets gate

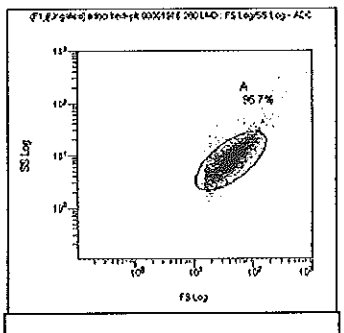


serum+ untreated platelets

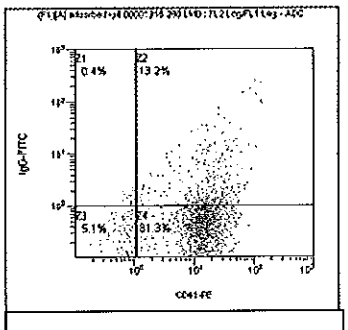


serum+chloroquine- treated platelets

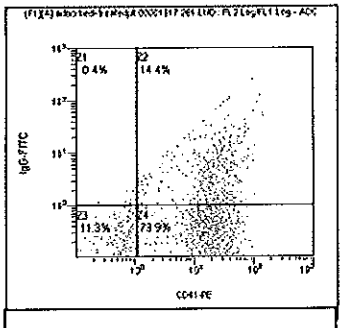
(B)



platelets gate



adsorbed serum+ untreated platelets



adsorbed serum+ chloroquine-treated platelets

2. Sensitivity and specificity analyses of the three assays

2.1 HLA antibody tests analyses

HLA antibody screening in 43 sera by LCT found positive antibodies 18 sera, negative reactions in 25 sera. SPRCA gave positive reaction in 17 out of 43 sera and 26 sera were negative with HLA antibody screening. Flow cytometry test gave positive 19 out of 43 sera and negative 24 sera (table 8).

When SPRCA compared with LCT, 16 sera showed positive results both LCT and SPRCA and 24 sera showed negative results in both of techniques. One serum showed a positive SPRCA and a negative LCT (patient 26). Two sera (patient 23, 37) showed negative results tested by SPRCA and positive by LCT. The sensitivity and specificity were calculated used formula as described in previous chapter. The sensitivity and specificity of SPRCA was 88.89% and 96.00%, respectively (table11).

Flow cytometry technique was compared with LCT showed 18 positive sera using LCT and flow cytometry techniques. Twenty-four sera showed negative reactions tested by both of assays. None sample showed a positive LCT and a negative flow cytometry. One serum (patient 39) showed a negative LCT and a positive flow cytometry initially. According to serum from patient 39 was confirmed for HLA antibodies and showed agreement results with flow cytometry, so none of the serum was false positive by flow cytometry technique. The sensitivity and specificity of flow cytometry was 100% and 100%, respectively (table 12).

Table 11. Sensitivity and specificity analyses of SPRCA when LCT was used as a reference technique. The sensitivity and specificity of SPRCA was 88.89% and 96.00%, respectively

		LCT		
		Positive	Negative	Total
SPRCA	Positive	16	1	17
	Negative	2	24	26
	Total	18	25	43

Table 12. Sensitivity and specificity analyses of flow cytometry technique when LCT was used as a reference technique. The sensitivity and specificity of flow cytometry was 100% and 100%, respectively

		LCT		
		Positive	Negative	Total
Flow cytometry	Positive	18	1*	19
	Negative	0	24	24
	Total	18	25	43
* Serum from patient 39 contained HLA antibody and was false negative by LCT				

Because of the high sensitivity and specificity, flow cytometry technique was considered to be a reference assay in HLA antibody screening. Sensitivity and specificity of LCT and SPRCA techniques were analyzed to assess the ability in HLA detection.

When LCT was compared with the flow cytometry technique, there were 18 positive sera using LCT and the flow cytomtry technique. Twenty-four sera showed negative reactions when tested using both of assays. None of the samples showed a negative flow cytometry and positive LCT result. Only one serum (patient 39) showed a positive flow cytometry and a negative LCT result. The sensitivity and specificity of LCT were 94.73 % and 100 %, respectively (table 13). These showed that LCT was of a lower sensitivity than the flow cytometry technique in HLA antibody detection.

The sensitivity and specificity of SPRCA was assessed when compared with flow cytometry technique. There were 16 sera showed positive both SPRCA and flow cytometry assay and 23 sera showed negative results in both of techniques. Only one serum was negative by flow cytometry assay and positive by SPRCA (patient 26). There were three sera (patient 23, 37 and 39) which showed positive results using the flow cytometry technique but negative using SPRCA. The sensitivity and specificity of SPRCA was 84.21 % and 95.83 %, respectively (table14).

Table 13. Sensitivity and specificity analyses of LCT when the flow cytometry technique was used as a reference technique. The sensitivity and specificity of LCT was 94.73% and 100%, respectively.

		Flow cytometry		
		Positive	Negative	Total
LCT	Positive	18	0	18
	Negative	1	24	25
	Total	19	24	43

Table 14. Sensitivity and specificity analyses of SPRCA when the flow cytometry technique was used as a reference technique. The sensitivity and specificity of SPRCA was 84.21 % and 95.83 %, respectively

		Flow cytometry		
		Positive	Negative	Total
SPRCA	Positive	16	1	17
	Negative	3	23	26
	Total	19	24	43

2.2 Screening for HPA antibodies

In case of screening for only HPA antibodies, all of data from table 7 were analysed to calculate sensitivity and specificity of SPRCA when flow cytometry technique was taken as a reference method. Flow cytometry techniques showed that 4 patients had reactive HPA antibody. Flow cytometry technique and SPRCA gave the reactive HPA antibodies in 2 patients. Thirty-nine sera were negative both techniques. Two sera showed negative by SPRCA but positive by flow cyometry technique. None of serum was positive by SPRCA and negative by flow cytometry (table 9). The sensitivity and specificity of SPRCA were calculated and the results were 50 % and 100 %, respectively (table 15).

Table 15. Sensitivity and specificity analyses of SPRCA when the flow cytometry technique was used as a reference technique. The sensitivity and specificity of SPRCA were calculated and the results were 50 % and 100 %, respectively.

		Flow cytometry		
		Positive	Negative	Total
SPRCA	Positive	2	0	2
	Negative	2	39	41
	Total	4	39	43

3. Comparison of results of platelet-reactive antibody screening test

3.1 Statistic analyses of the tests

The concordance of LCT, SPRCA and flow cytometry was analyzed statistically using data from 10 healthy donors and 43 patients (table 6 and 7). Correlation and strength of

concordance between each techniques was calculated with the SPSS version 16.0 software program. For every combination of two techniques, a 2 x 2 table was generated and chi square test (χ^2) was analyzed for independence. For the strength of concordance, a Kappa coefficient (K) was determined. The results of LCT and the flow cytometry technique were significantly correlated ($p<0.001$, $K = 0.805$) (table 16-A), and the results of SPRCA and flow cytometry were also correlated significantly ($p<0.001$, $K = 0.761$) (table 16-B). Similar to the other combinations of two techniques, LCT and SPRCA were also significantly correlated ($p<0.001$, $K = 0.754$) (table 16-C).

An agreement between each pairing of methods was also compared by calculation. The agreement result between LCT and flow cytometry was 90.56%, SPRCA and flow cytometry was 88.68%, LCT and SPRCA was 90.56%. Disagreement in results between LCT and flow cytometry was 9.44%, SPRCA and flow cytometry was 11.32%, LCT and SPRCA was 9.44% (table 16 and 17).

Table 16. Comparison of results of the platelet-reactive antibody screening test: LCT versus flow cytometry (A), SPRCA versus flow cytometry (B), and LCT versus SPRCA (C)

		Flow cytometry		
		Positive	Negative	Total
LCT	Positive	18	0	18
	Negative	5	30	35
	Total	23	30	53
χ^2=34.822; p<0.001 K = 0.805				

Table 16-A. LCT versus flow cytometry

Correlation of the flow cytometry technique and LCT was analyzed, the results of the flow cytometry technique were correlated significantly with results of LCT (χ^2=34.822; $p<0.001$) with a good strength of agreement ($K = 0.805$).

		Flow cytometry		
		Positive	Negative	Total
SPRCA	Positive	18	1	19
	Negative	5	29	34
	Total	23	30	53
χ^2=34.822; p<0.001 K = 0.761				

Table 16-B. SPRCA versus flow cytometry

Correlation of flow cytometry and SPRCA was analyzed, the results of the SPRCA technique were correlated significantly with the results of flow cytometry (χ^2=34.822; p<0.001) and a good strength of agreement K = 0.761).

		SPRCA		
		Positive	Negative	Total
LCT	Positive	16	2	18
	Negative	3	32	35
	Total	19	34	53
χ^2=30.121; p<0.001 K = 0.754				

Table16-C. LCT versus SPRCA

Correlation of SPRCA and LCT was analyzed, the results of the SPRCA technique were correlated significantly with the results of LCT (χ^2=30.121; p<0.001) and a good strength of agreement (K = 0.754).

Table17. Agreement and disagreement of results tested by three methods

Methods / Agreement	LCT vs flow cytometry	SPRCA vs flow cytometry	LCT vs SPRCA
Agreement	90.56% (48/53)	88.68% (47/53)	90.56% (48/53)
Disagreement	9.44% (5/53)	11.32% (6/53)	9.44% (5/53)

Agreement = positive or negative results with both tests matched (positive-positive or negative-negative)

Disagreement = results obtained with the two tests were opposite (positive-negative or negative-positive)

4. Comparison of cost and time consumption in LCT, SPRCA and flow cytometry

The procedural steps, cost and time used for each techniques were summarized in table18. Time consumption of LCT and SPRCA were 8 and 6 hours, respectively. The flow cytometry technique needed a shorter amount of time to perform only for 4 hours. The cost of LCT and SPRCA was 746 and 735 Baht respectively, and the cost of the flow cytometry technique was 1,661 Baht. Total cost and time consumption of LCT, SPRCA and flow cytometry were summarized in table19.

Table18. Calculated cost, labor and time used for performing these tests; LCT, SPRCA and flow cytometry on one patient's serum tested against six screening cells.

Calculated cost, labor and time used for performing LCT

Procedure steps	Cost	Time consumption
1. Blood collection	51.00 Baht	30 minutes
2. Lymphocyte separation	411.00 Baht	85 minutes
3. Incubation of cell-serum in Terasaki tray	169.00 Baht	90 minutes
4.Stopping and reading the reactions	35.00 Baht	4 hour 25 minutes
5. Power expense and labor expense	80.00 Baht	-
Total	**746.00 Baht**	**8 hours**

Calculated cost, labor and time used for performing SPRCA

Procedure steps	Cost	Time consumption
1. Microtiter plate coating	260.50 Baht	2 hours 20 minutes
2. Blood collection	51.00 Baht	30 minutes
3. Platelet preparation	223.00 Baht	110 minutes
3. Incubation of cell-serum	60.50 Baht	65 minutes
4.Stopping and reading the reactions	10.00 Baht	15 minutes
5. Power expense and labor expense	130.00 Baht	-
Total	**735.00 Baht**	**6 hours**

Calculated cost, labor and time used for performing flow cytometry technique

Procedure steps	Cost	Time consumption
1. Blood collection	51.00 Baht	30 minutes
2. Platelet preparation	158.00 Baht	90 minutes
3. Incubation of platelets and serum	285.00 Baht	80 minutes
4. Flow cytometry analysis	1,035.00 Baht	40 minutes
5. Power expense and labor expense	132.00 Baht	-
Total	**1,661.00 Baht**	**4 hours**

Table 19. Summary of total cost and time consumption between LCT, SPRCA and flow cytometry

Tecniques	Total cost (Baht)	Time (hour)
LCT	746	8.0
SPRCA	735	6.0
Flow cytometry	1,661	4.0

CHAPTER 4

DISCUSSION

This study was to determine the presence of platelet-reactive auto and alloantibodies in patients' sera comparing between three techniques which were LCT, SPRCA and flow cytometry assay. The data showed that LCT, SPRCA and flow cytometry demonstrated a similar in testing results with very few in differences. The study in healthy control group who were blood donors and had never been transfused or pregnancy, platelet-reactive antibodies were not observed in all three methods. In the patient study 19 out of 43 sera similarly were found non reactive tested by all three methods. There were 24 out of 43 sera reactive against either HLA or HPA antigens in all three methods. The reactive antibody against HLA were found similarly in 16 out of 24 sera tested by all three methods and 4 out of 24 sera had discrepancy results. One of the discrepancy serum was from the ITP patient (patient 39) and the primary result was HLA reactive by only flow cytometr. According to the limited most sensitive MAIPA in our laboratory and in Thailand the confirmation test of HLA reactive antibody was performed against HLA antigens on mononuclear cells and it was found positive reaction. This test was designed according to other studies reported the high expression of HLA molecules on mononuclear cells than the expression on platelets (Liebert et al., 1997 and Kiefel et al., 2001). This confirmed study demonstrated that the negative reactions by LCT and SPRCA were false negative. In patients no. 23 and 37 the results were HLA antibody reactive tested by both flow cytometry and LCT but non reactive by SPRCA. This was likely true positive as several previous studies had been showed low sensitivity of SPRCA (Freedman et al., 1996 and Harrison et al., 2003). In patient no.26 the serum was weakly reactive in two out of six target platelets using SPRCA method but were non reactive when tested by both flow cytometry and LCT. This was likely false reactive because HLA expression on platelet was not as strong as the expression on mononuclear cells (Liebert et al., 1997 and Kiefel et al., 2001) and SPRCA detects HLA reactive antibody on platelet while LCT detects HLA reactive antibodies on mononuclear cells. In our study when considered that flow cytometry was the highest sensitive technique there were 19 out of 43 sera positive to HLA auto- and alloantibody. According to this study when ITP subjects who had diseases associated with autoantibody were not taken into account (13 subjects), HLA alloimmunisation was a major clinical problem (60%)

in patients who had problem with platelet refractoriness. This information was consistent with other studies which found HLA alloimmunisation around 30-70% (Kurz et al.; Kiefel et al. and Sanz et al., 2001).

The reactive antibody against HPA tested by flow cytometry and SPCA were found similar in 2 out of 43 sera and discrepancies were found in 2 out of 43 sera. One of the discrepancy was in the patient no. 43 who was the mother of a neonatal thrombocytopenic baby and the reactives were found in target platelets both untreated (contained HLA and HPA) and chloroquine treated platelets (contained only HPA). The cause of thrombocytopenia was unknown whether from HLA or HPA antibodies. The test was designed to differentiated between HLA and HPA antibodies by absorption HPA antibody from the serum using cholroquine treated platelets. Then the remain absorbed serum was re-analysed by flow cytometry technique using both untreated and cholroquine treated platelets and the analysis showed that there was not HLA and HPA antibodies left in the absorbed serum. This information confirmed that the causative antibody was the sole HPA antibody. In addition, serum from mother was crossmatched against father's platelets and the reaction was positive. This data confirmed that alloantibodies of mother against father's platelets. Another discrepancy serum was from ITP patient that the reactive was found only in flow cytometry but negative by SPRCA. The confirmation test was not performed as serum was unavailable but several studies suggested the HPA antibody was the autoantibody found in ITP (Berchtold et al., 1993; He et al., 1994 and Biglino et al., 1997). According to the most sensitive MAIPA is not available in our laboratory therefore flow cytometry was considered to be very sensitive approaching to MAIPA ((Allen et at., 1994; Kohler et al., 1996 and International Forum, 2003). It was likely that patient no. 42, 43 were false negative by SPRCA.

In this study flow cytometry demonstrated highest sensitivity compare to LCT and SPRCA. There was a patient who was positive by flow cytometry but false negative by LCT (patient no.39 who was ITP). The explanation could be that LCT detected cytotoxicity as a result of the presence of complement-activating HLA antibodies bound on lymphocytes, whereas the SPRCA and flow cytometry techniques detected platelet-reactive IgG antibodies bound on platelets. Sera from HLA alloimmunised patients tested against a panel of either lymphocytes or platelets from random donors do not always show the concordant results as seen in different numbers of reactive antibodies against lymphocytes and platelets in each panel. There were 13

patients that the reactive antibodies against number of lymphocytes and platelets in each panel were different such as in patient no. 20,22,23, 25,27,28,30,31,33,34,35,37 and 38. This possibly explained that antibodies to HLA class I antigens may not be able to activate complements, or mixtures of HLA antibodies may contain portions that are able to bind to certain HLA molecules without the capacity to activate complements. On the other hand, sera were found that react in the LCT, but that did not contain antibodies binding to HLA class I antigens. This can be occurred in patients who have antibodies reacting with structures on lymphocytes or other than HLA class I antigens in their sera. Another reason for discrepancies between LCT results and immunoglobulin-binding assays with platelets and lymphocytes from the same donors may be the variable and sometimes low expression of certain HLA class I antigens on platelets (Kiefel et al., 2001). Liebert and Aster demonstrated that HLA-B12 is expressed on platelets at densities varying $\pm$ 35 times from lowest to highest density (Liebert and Aster, 1977).

HLA reactive antibodies were weak by LCT and the flow cytometry technique, while SPRCA showed negative reactions (patient 23 and 37). The possible cause was HLA class I molecules on lymphocytes which were target cells for LCT were greater than on platelets which were target cells for SPRCA. Patient 39 showed a positive result when using flow cytometry only using untreated platelets and a negative one when using LCT and SPRCA. This was probably caused by HLA antibodies in the serum had low activity for complement fixation resulting in the LCT showing a negative reaction. In addition, the flow cytometry technique was capable for detecting very small numbers of antibody molecules bound to HLA class I molecules on target platelets. We confirmed the HLA antibodies using mononuclear cells as target cells and flow cytometry analysis was done. Flow cytometry analysis showed a positive result with high average fluorescence intensity, so this serum contained HLA antibodies but LCT and SPRC were unable to detect. False negative result of LCT in this case caused the lower sensitivity when compare to the flow cytometry technique.

In two sera of patient 40 and 41, LCT demontrated negative reaction while SPRCA and the flow cytometry technique gave positive reaction in both chloroquine-treated and untreated platelets. Sera that were positive in both chloroquine-treated and untreated platelets could be the result of antibodies direct against HPA alone or HPA plus HLA antibodies in combination. Because of clinical presentation and patient history, two sera contained HPA

antibodies. Patient 40 was confirmed by the HPA antibody identification test against platelet panel cells using SPRCA as tested by the Thai National Blood Centre and the result showed anti-HPA-1a antibody. The positive reactions found by flow cytometry were probably caused by greater sensitivity for platelet reactive antibodies in the flow cytometry technique.

Patient 42 and 43, showed negative reaction when tested by LCT and SPRCA and showed a positive reaction only when using the flow cytometry technique in both chloroquine-treated and untreated platelets. Again, this could be caused by antibodies directed against HPA. The probable reason for the negative SPRCA findings was the antibodies targeting a labile component of HPA antigens such as HPA-3b (Harrison et al., 2003 and Kataoka et al., 2004) or targeting small numbers of molecules on the platelet membrane such as HPA-5 (1,000-2,000 sites per platelet) (MacFarland, 2003). Target cells for the flow cytometry technique were intact platelets and fresh preparation may maintain these antigens whereas lysate platelets were used in SPRCA which may be destroyed some of the HPA antigens (Harrison et al., 2003).

Patient 43, a mother who delivered a baby suspected NAIT, was studied to investigate the antibodies either direct targeting HPA alone or HPA plus HLA antibody in combination. The serum was absorbed with chloroquine-treated platelets. Then the absorbed serum was tested with untreated platelets and chloroquine-treated platelets using the flow cytometry technique. The results proved negative in both of the platelet panels, indicating the antibodies in serum was targeting HPA alone (figure 10). Several reports (Berchtold et al., 1993; He et al., 1994 and Biglino et al., 1997) demonstrated that in ITP patients the possible abnormal antibody was HPA antibody. Our study had scarce samples with HPA antibody therefore subjects with platelet autoantibody were recruited.

The sensitivity and specificity of techniques used for platelet reactive antibody detection were studied by investigators in Europe, and there they found that the MAIPA was the highest sensitive and specific (International Forum, 2003). MAIPA was taken as a reference method in many reference laboratories, but it was more expensive, complicated in procedure performed and time-consuming. Flow cytometry was considered as reference technique because the sensitivity and specificity were approaching to MAIPA technique. This was also observed in two British Workshops (Allen et at., 1994). Kohler et al. (1996) found that flow cytometry technique had nearly the same sensitivity and specificity as MAIPA and were 94.7 % and 96.3 %,

respectively. In recent reports, the flow cytometry technique was taken as a reference method in establishment of modification of SPRCA (Vongchan et al., 2008).

When comparing SPRCA and the flow cytometry technique with LCT for screening only for HLA antibodies, the data showed that flow cytometry was more sensitive and specific than the SPRCA techniques. Flow cytometry gave 100 % sensitivity and 100% specificity, whereas SPRCA gave 88.89 % sensitivity and 96.00% specificity. Because of patient 39 whose serum was confirmed to contained HLA antibody, LCT gave a false negative result. When the flow cytometry technique was a reference for HLA antibody screening, LCT showed only 94.73% sensitivity and 100% specificity. The data showed that the flow cytometry technique was more sensitive than LCT with no difference in specificity. This agrees with other authors' reported that flow cytometry assay was more sensitive than LCT for detection of HLA antibodies (Moses et al., 2000; Levin et al., 2003 and Sato et al., 2005).

Sensitivity and specificity for platelet-specific antibody screening of SPRCA were poor sensitivity (50%) with equivalent specificity (100%). This is possible from too low number of samples and the calculated sensitivity may not accurate. Anti-HPA antibodies were observed in only 4 patient samples. The statistical analysis of sensitivity of SPRCA for HPA screening may not be precise. For the future studies, the number of samples with anti-HPA antibodies should be large enough to provide an adequate power of conclusion.

SPRCA was less sensitive when compared with other techniques, several researchers tried to improve them using some modified techniques. The technique to increase the sensitivity of the SPRCA were studied by Fogg. Pretreatment of reagent platelets with enzymes increased the reactivity of known antibodies and detected some HLA and HPA antibodies (Fogg, 2002). Moreover, Vongchan et al modified SPRCA to reduce labor expense and to increase cost effectiveness. They performed SPRCA using a different form of anti-platelet antibody to immobilized HLA or HPA to the microtiter plate. Another modified procedure was replacing intact platelets with platelet lysate. Through these modifications, the sensitivity was increasing to 98 % and specificity to 91 % when flow cytometry was a referent (Vongchan et al., 2008).

When analyzing all samples for both HLA and HPA antibody screening, LCT and flow cytometry showed the techniques to be concordant (90.56% agreement, $\kappa = 0.805$); SPRCA and flow cytometry were in concordance (88.68 % agreement, $\kappa = 0.761$); SPRCA and

LCT were also in good agreement (90.56% agreement, $K = 0.754$, respectively). Comparison of correlation using chi-square test showed significant correlation in all pair of techniques ($p<0.001$) (table 16-A, -B, -C). These data suggested that LCT had better concordance results than SPRCA when compared with the flow cytometry technique. However, LCT detected only HLA antibodies on lymphocytes but did not on platelets. These data were in consistent with other studies (Worfork et al., 1991 and Levin et al., 2003).

Comparison of the cost and time necessary for the three difference techniques showed that LCT and SPRCA were similar in cost and time-consumption, but SPRCA was more complicated than LCT. Flow cytometry technique was the highest cost but lowest time-consuming. Each SPRCA and the flow cytometry techniques could detect both HLA antibodies and HPA antibodies whereas LCT detected only HLA antibodies. Because of the simultaneous incubation of the serum with chloroquine-treated and untreated platelets, antibodies of HLA and HPA could be detected in the same experiment Moreover, platelet screening cells for SPRCA could be frozen until use, but lymphocytes preparations need to be from fresh blood. Similar to the target cells of LCT which requires fresh lymphocytes, the flow cytometry technique needed intact platelets as the target cells. The advantages and disadvantages of LCT, SPRCA and the flow cytometry techniques were summarized in table 20.

There were two patients whom provisional diagnoses were NAIT and idiopathic thrombocytopenia that SPRCA could not detect HPA antibodies while sera were reactive with flow cytometry. This is crucial that SPRCA was not sensitive enough to diagnose NAIT of which the risk of a fetus/newborn is too high. According to our study SPRCA should not be used for NAIT testing. SPRCA may have a role in some places where flow cytometry test is not available. SPRCA may be used for platelet crossmatching as it does not need expensive instrument. In our study we found that LCT test and SPRCA had lower sensitivity compare to flow cytometry. These detections did not have crucial effect for platelet crossmatching but these may have some effects on HLA antibody screening and crossmatching in potential organ transplant recipients which LCT is commonly used in Thailand.

Table 20. Summary of the advantages and disadvantages of LCT, SPRCA and flow cytometry techniques when considered to apply as routine laboratory tests

Advantages			Disadvantages		
LCT	**SPRCA**	**Flow cytometry**	**LCT**	**SPRCA**	**Flow cytometry**
1. Capable of detecting only HLA class I antibody	1. Capable of detecting HLA and HPA antibodies in the same experiment	1. Capable of detecting HLA and HPA antibodies in the same experiment including antibodies targeting labile HPA	1. Unable to detect HPA antibodies	1. Limitations in detecting HPA antibodies targeting labile HPA antigens	1. Capable of detecting very small numbers of antibody molecules (non specific antibody) bound to platelets that may cause high background signals
2.Target cells were lymphocytes which bear large numbers of HLA class I molecules	2. Target cells for antibody screening could be frozen until used	2. Target cells were from fresh preparations, all of the HPA antigens were still on platelets	2. Target cells were lymphocytes with no HPA antigens	2. Target cells were platelet lysates that may destroy labile HPA antigens	2. Required only intact platelet as a target cells
3. Small volume of sera were required	3. Simple to drop the sera into the microplate	3. Small volume of sera were required	3. special skills to apply small sera volumes were required	3. Large volumes of sera were required	3. Platelet target cells must be blocked with fetal calf serum before use

Table 20. Summary of the advantages and disadvantages of LCT, SPRCA and flow cytometry techniques when considered to apply as routine laboratory tests (continue)

Advantages			Disadvantages		
LCT	**SPRCA**	**Flow cytometry**	**LCT**	**SPRCA**	**Flow cytometry**
4. Inexpensive reagents and instruments were required	4. Inexpensive reagents and instruments were required	4. Reading of the reaction was simple and objective	4. Not easy to apply to routine use and reading of the reaction was subjective	4. Not easy to apply to routine use and reading of the reaction was subjective	4. Flow cytometer was required
5. Lower cost	5. Lower cost	5. Speedy	5.Time-consuming	5. Time-consuming	5. More expensive
6. High sensitivity and specificity for HLA antibody detection	6. Slightly high sensitivity and specificity for HLA and HPA antibody detection	6. High sensitivity and specificity for HLA and HPA antibody detection	6. Limitation to differentiating HPA antibodies when present together with HLA antibodies	6. Limitation to differentiating HPA antibodies when present together with HLA antibodies	6. Limitation to differentiating HPA antibodies when present together with HLA antibodies

CHAPTER 5

CONCLUSION

Flow cytometry had been shown to be the highest sensitivity and specificity technique compare to LCT and SPRCA for platelet reactive antibodies both HLA and HPA antibodies detection. Although it has higher expensive cost from FITC-labeled monoclonal antibody against human IgG and instrument than those used in LCT and SPRCA techniques. Flow cytometer was used to measure the fluorescence intensities of the tests this was more objective than microscopic evaluation. However, there were significantly correlated between these three techniques and good strength of agreement in overall, most of positive and negative results were concordance. It was the highly sensitive, very specific, rapid, requiring a small amount of serum, easy to perform at low cost and simple to interpret. In summary, consistent with previous reports, the flow cytometry technique was considered to establish the proper methods suitable for routine investigations of alloimmune causes of platelet transfusion refractoriness and including NAIT and PTP.

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จรินทร์ บัวแก้ว, จารุพร พรหมวงศ์. การศึกษาเปรียบเทียบความไวและความจำเพาะของการทดสอบในการตรวจหาแอนติบอดีที่จำเพาะต่อเกล็ดเลือด. นำเสนอผลงานด้วยโปัสเตอร์ในการประชุมวิชาการงานบริการโลหิตระดับชาติ วันที่ 19-20 มีนาคม 2552 ณ โรงแรมโซฟิเทล เซ็นทาราแกรนด์ กรุงเทพมหานคร

Oral presentation

จรินทร์ บัวแก้ว, จารุพร พรหมวงศ์. เพิ่มความไวในการทดสอบหาแอนติบอดีต่อเกล็ดเลือดโดยใช้เทคนิค flow cytometry. การประชุมวิชาการคณะแพทยศาสตร์ ประจำปี 2552 วันที่ 5-7 สิงหาคม 2552 ณ อาคารเรียนรวมและหอสมุดคณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์