A PREVALENCE STUDY OF PLATELET ALLOANTIBODIES IN MULTIPLY TRANSFUSED THROMBOCYTOPENIC PATIENTS IN HUSM

by

Dr WAN HASLINDAWANI WAN MAHMOOD

M.D.(USM)

Dissertation Submitted In Partial Fullfillment Of The Requirements

For Degree Of Masters Of Pathology (Haematology)

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Dedicated to my lovely husband, Suhairi Ibrahim and my two daughters Nur Syazlin Amalina and Nur Sofea Amirah for supporting me and loving me unconditionally.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACD	Acid-Citrate Dextrose
ACE	Angiotensin- Converting Enzyme
Ag	Antigen
AIHA	Auto-Immune Haemolytic Anaemia
BFU	Burst- Forming Unit
CD	Cluster of Differentiation
CPDA-1	Citrate- Phosphate-Dextrose- Adenine 1
CPD	Citrate-Phosphate-Dextrose
EDTA	Ethylenediamine Tetra-Acetic Acid
ELISA	Enzyme Linked Immuno-Sorbant Assay
FBP	Full Blood Picture
FFP	Fresh Frozen Plasma
FNHTR	Febrile Non-Haemolytic Transfusion Reaction
GP	Glycoprotein
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HPA	Human Platelet Antigens
HTR	Haemolytic Transfusion Reaction
ITP	Idiopathic Thrombocytopenic Purpura

LISS	Low Ionic Strength Saline
NAIT	Neonatal Alloimmune Thrombocytopenia
PTP	Post-Transfusion Purpura
PTR	Post-Transfusion Refractoriness
SLE	Systemic Lupus Erythematosus
TAGvHD	Transfusion Associated Graft Versus Host Disease
TRALI	Transfusion Associated Lung Injury
RBC	Red Blood Cell
WBC	White Blood Cell

ABSTRAK

Pesakit yang kerap menerima pemindahan darah adalah terdedah kepada platelet alloimmunisasi. Alloantibodi kepada platelet akan menyebabkan kegagalan kepada transfusi platelet. Tujuan kajian ini dijalankan adalah untuk mengesan alloantibodi kepada platelet didalam pesakit yang kerap menerima transfusi darah di HUSM.

Sebanyak sembilan puluh lima pesakit thrombocytopenia (kiraan platelet < 100 x 100^9/l) yang kerap menerima transfusi darah (lebih dari dua transfusi darah dari sebarang komponen dan tarikh terakhir pemindahan sekurang-kurangnya dua minggu dari tarikh kajian) diambil secara prospektif. Sampel darah diuji dengan menggunakan "Solid Phase System" (Capture-P).

Seramai 45 lelaki (47.4%) dan 50 perempuan (52.6%) termasuk dalam kajian ini. Jarak umur adalah diantara 3 hingga 90 tahun. Kekerapan transfusi darah adalah diantara 10 hingga 168 kali. Tujuh pesakit (7.4%) didapati alloantibodi kepada platelet, terutamanya anti-HPA-5b (4.2%). Tiga pesakit (3.2%) daripada mereka menunjukkan paten yang tidak khusus. Enam pesakit (6.3%) telah menerima transfusi sel darah merah padat kurang daripada 20 unit dan seorang pesakit (1.1%) telah menerima transfusi lebih daripada 20 unit sel darah merah padat. Empat pesakit (4.2%) menerima transfusi platelet kurang dari 20 unit dan tiga pesakit (3.2%) menerima lebih dari 20 unit platelet.

keputusan ini membuktikan implikasi di masa hadapan dalam memilih penderma platelet yang mempunyai alloimmunisasi.

ABSTRACT

Multiply transfused patients are frequently subjected to platelet alloimmunization.These platelet alloantibodies produced can result in refractoriness to platelet transfusion. The aim of this study is to detect the presence of anti-platelet alloantibodies in multiply transfused thrombocytopenic patients in HUSM

Ninety five thrombocytopenic (platelet count <100 x 10^9/l) and multilply transfused (more than 2 times transfusion of any blood component with last transfusion more than 2 weeks prior to the study) patients were recruited prospectively. The blood samples were tested using a Solid Phase system (Capture P).

There were 45 males (47.4%) and 50 females (52.6%) recruited with ages from 3 to 90 years. The frequency of transfusions ranged from 10-168. Seven patients (7.4%) were detected to have platelet alloantibodies, predominantly anti-HPA-5b in 4 patients (4.2%). Three of them (3.2%) showed a non-specific pattern. Six patients (6.3%) had received packed cells less than 20 units and another 1 (1.1%) received more than 20 units of packed cells. Four patients (4.2%) received platelet transfusion of less than 20 units and another 3 patients (3.2%) received more than 20 unit platelets.

The study may have future implications for the selection of platelet donors for alloimmunized recipients in HUSM.

Chapter 1

INTRODUCTION

1.0 INTRODUCTION

1.1. Platelets

1.1.1. Formation of blood platelets

Blood platelets are fragments of the cytoplasm of megakaryocytes, hence there are non nucleated and are formed chiefly in the bone marrow. Most of the available knowledge about thrombopoeisis is derived from bone marrow culture experiments, or from observations in humans and animals recovering from drug induced thrombocytopenia, which are conditions under which physiological mechanisms may not prevail.

Megakaryocytes are derived from pluripotential stem cells, as the earliest recognized platelet precursors is a burst-forming unit denoted BFU-Meg. Under the influence of thrombopoeitin (Tpo) and cytokines such as IL-3 and IL-11, the BFU-Meg develop into megakaryocyte colony-forming units (CFU-Meg).

The committed progenitor cells cease to undergo classical mitosis, developing instead by endomitotic reduplicative, where nuclei divide but not the cell, the morphological and biochemical features of megakaryoblasts and then megakaryocytes. Their ploidy states progress from 2N up to 32N or even 64N. Their diameters increase from 6-20μm (megakaryoblasts) to around 60μm (fully mature megakaryocyte) associated with proliferation of the characteristic platelet granules (α-granules and dense bodies) and

membrane glycoproteins, which are vital to platelet functions. The membrane glycoproteins have different functions for example glycoprotein IIb-IIIa involves in platelet aggregations and glycoprotein Ib involves in platelet adhesions to subendothelial microfibrills.

The rapid increase in cytoplasm is accommodated by progressive folding, or invagination, of its membrane and it has been proposed, that this process accounts for the appearance of demarcation membranes, which eventually form the boundary of individual platelets. A fully mature megakaryocyte begins to extend pseudopodia, which penetrate through the walls of adjacent individual platelets or larger cytoplasmic fragments. The latter 'proplatelets' are transported through the bloodstream via the heart to the lungs, where final mechanical fragmentation is accomplished in the pulmonary microcirculation. Each megakaryocyte gives rise to as many as 3000 platelets.

Around 20-30% of 'circulating' platelets appear to be sequestered in the spleen, although it is not known if the splenic pool represents the newly released cells. The normal life span of platelets ranges between 8 and 14 days. The platelet count in the peripheral blood is in the range 150-400 x 10^9/l in normal subjects. (Hoffbrand et al.,1999)

1.1.2. Structure and function of the blood platelets.

Platelets that have been fixed immediately after removal from the body appear under the electron microscope as smooth, biconvex discs with a diameter of 2-4μm.

The platelet plasma membrane is vital to the cells function for two reasons. Firstly, it contains a number of specific glycoprotein (GP) receptors, through which platelets interact with aggregating agents, inhibitors, coagulation factors (such as fibrinogen, von Willibrand factor and thrombin) and consequently, with the vessel wall and with each other. Secondly, the platelet membrane also contains phospholipids, which are concerned with prostaglandin synthesis and calcium mobilization within the cell, and with the generation and localization of procoagulant activity on the outer surface of the platelet. (Hoffbrand et al., 1999)

1.2 Blood products

A blood product is defined as any therapeutic substance prepared from human blood. Whole blood is unseparated blood collected into an approved container containing an anticoagulant-preservative solution. Whole blood is obtained from human blood donors by venesection. Whole blood may be suitable for transfusion in many clinical situations, such as red cells replacement in acute blood loss where there is also hypovolaemia. However, the whole blood is also a source of other important components and the separation of whole blood into its constituent component-red cells, platelets and plasma is widely practiced for use when these components only are specifically required.

A blood component is a constituent of blood, separated from whole blood into red cell concentrates, plasma and plasma components such as fresh frozen plasma (FFP) and cryoprecipitate as well as platelet concentrate.
(Emmanuel, 2001)

For platelet concentrates, there are two types which are available for transfusion:

1. platelet from random donor
2. apheresis platelet.

Platelets are prepared from whole blood of apparently healthy donors by differential centrifugation, while apheresis platelets are harvested from individual donors through apheresis with minimal or no contamination by leucocytes or red cells. Although platelets are the predominant cellular component, both products also contain significant amounts of red cells, leucocytes and plasma. All these blood components in platelet concentrates are potentially immunogenic and can lead to various immunological and clinical consequences in transfusion recipients. (Kao et al., 2000)

1.3. Blood transfusion

1.3.1. Indications

Blood transfusion involves the infusion of whole blood or the blood components from one individual (the donor) to another (the recipient).

For the whole blood, it is usually reserved for red cell replacement in acute blood loss with hypovolaemia, for example in traumatic or surgical blood loss, or severe gastrointestinal or uterine haemorrhage. It is also used in exchange transfusion for the case of neonatal jaundice. Sometimes it is given to the patients needing red cell transfusions where red cell concentrates are not available.

Red cell concentrates are the treatment of choice in chronically anaemic patients who require transfusion. In older subjects, a diuretic is often given simultaneously and the infusion should be sufficiently slow to avoid circulatory overload. In the majority of patients with deficiency anaemias appropriate therapy with iron, folate or vitamin B_{12} is sufficient and red cell transfusions are seldom required. In chronic anaemias which do not respond to haematinics, transfusion should be avoided unless the patient is at risk from, or incapacitated by, the anaemia. This concentrates is also used with crystalloid replacement fluids or colloid solution in acute blood loss.

Platelet concentrates as mentioned earlier are harvested by cell separators or from individual donor units of blood. These concentrates are indicated in severely thrombocytopenic patients or patients with platelet dysfunctions in established haemorrhage or prophylactically to prevent bleeding for example during intensive myelotoxic chemotherapy to keep the platelet count above 5-10 X 10^9/l. Their most important use is therefore in the support of patients with severe bone marrow failure for example due to acute leukaemia, aplastic anaemia, myelodysplasia or bone marrow transplantation. If fever, infection or concurrent coagulopathy is present or the platelet count is falling rapidly or potential bleeding sites for example surgical wounds are present, the platelet count should be kept more than 20 X 10^9/l. Platelets may also be needed for patients with platelet functional disorders, following massive blood transfusion during cardiopulmonary by-pass surgery with bleeding not due to surgically correctable disorders and for patients with disseminated intravascular coagulation and bleeding. Platelets are not usually used in immune thrombocytopenias but may be given in auto-immune thrombocytopenia with major haemorrhage and in neonatal alloimmune thrombocytopenia.

For the fresh frozen plasma, it is a rapidly frozen plasma separated from fresh blood and its main use is for the replacement of coagulation factors, for example when specific concentrates are unavailable or after massive transfusions, liver disease, warfarin anticoagulant overdose and disseminated intravascular coagulation (DIC). It is also indicated in thrombotic thrombocytopenic purpura (TTP).

Cryoprecipitate is obtained by thawing fresh frozen plasma at 4°C and contained factor VIII, factor XIII, von Willebrand factors (vWF), fibronectin and fibrinogen. It is used widely as replacement therapy in haemophilia A and von willebrand's disease before more purified preparations of factor VIII became available. It is also used as a source of fibrinogen in acquired coagulopathies eg DIC. (Emmanuel, 2001)

1.3.2. Adverse effects of blood transfusion

Transfusion reactions occur in 7-10% of all recipients of blood or blood components. The majority of them are minor reactions, of which 10% are hemolytic and 90% are non hemolytic reactions. Transfusion reactions may be divided into acute (< 24 hours) and delayed (> 24 hours), as shown in Table 1.0 and Table 1.2.

Table 1.1 Acute Transfusion Reactions.

Immunologic	**Etiology**
Haemolytic transfusion reactions (HTR	ABO incompatibility
Febrile non-haemolytic transfusion reaction (FNHTR)	Cytokines ,antileucocyte antibodies
Allergic	Antibodies to plasma protein
Anaphylaxis	Antibodies to IgA
Transfusion related acute lung injury (TRALI)	Antibodies to leucocytes or to complement activation
Non Immunologic	
Marked fever with shock	Bacterial contamination
Atypical reaction with hypotension	Associated with ACE inhibitors
Congestive cardiac failure	Volume overload
Air embolism	Air infusion via line
Hypocalcaemia	Citrate toxicity

Hypothermia	Rapid infusion of cold blood
Hypokalaemia and hyperkalaemia	Red cell storage

Table 1.2 Delayed adverse reaction to transfusion (>24 hours).

Immunologic	**Etiology**
Alloimmunization to red blood cell (RBC), white blood cell (WBC) and platelets	Exposure to antigen of donor origin-plasma protein, human leucocyte antigen (HLA)
Hemolytic	Anamnestic antibodies to RBC antigens
Transfusion associated graft versus host disease (TAGvHD)	Engraftment of transfused functional lymphocytes Not well understood
Post transfusion purpura	Antiplatelet antibodies
Non Immunologic	
Iron overload	Multiple transfusion
Transfusion transmitted disease	Hepatitis, human immunodeficiency virus (HIV), Cytomegalovirus etc

(Emmanuel, 2001)

1.4. Causes of thrombocytopenia

Thrombocytopenia is defined as a platelet count of less than 150 X 10^9/l. There are many causes of thrombocytopenia which can be divided into non-immune thrombocytopenia and immune thrombocytopenia as shown in Table 1.3.

Table 1.3 Causes of thrombocytopenia.

A. Non immune thrombocytopenia is associated with :
-bone marrow transplant
-sepsis
-disseminated intravascular coagulopathy
-splenomegaly
-fever
-leukaemia/anaemia
-antibiotic therapy
-clinical bleeding
-hepatomegaly
-liver disease
-chemotherapy
-heart valves replacement

B. Immune thrombocytopenia:

1. Autoimmune thrombocytopenia this occurs because of the presence of autoantibodies to the patient's own platelet as in idiopathic thrombocytopenic purpura and acute thrombocytopenic purpura.

2. Alloimmune thrombocytopenic purpura which may be associated with multiple exposures to similar cellular antigens as in post transfusion purpura and neonatal alloimmune thrombocytopenia.

3. Drug induced which may occur following the administration of heparin, quinine, sulpha drugs and others.

1.5. Platelet alloantigens and alloantibodies.

Platelet alloantigens are defined by alloantibodies directed against genetically determined molecular variants of proteins or carbohydrates on the platelet membrane. The alloantibodies are elicited in normal individuals upon exposure to the alloantigen usually during pregnancy and in blood transfusion, or, rarely, by bone marrow transplantation. These alloantibodies bind to the target platelet alloantigens, resulting in immuno-mediated platelet phagocytosis.

Three major group of alloantigens are present on platelets:

1. Blood group antigens
2. Platelet-specific antigens
3. HLA's

There are different types of clinically relevant platelet alloantigens. Alloantigens shared with other blood cells and tissues are referred to as Type I antigens. Among these are the glycoconjugates of the blood group ABH system and the highly polymorphic HLA class I molecules. Type II alloantigens are more or less specific to platelets and are conventionally called platelet-specific alloantigens. It is well documented that platelet-specific antibodies against type II alloantigens play a role in the pathological mechanism of neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP) and post-transfusion refractoriness (PTR). In contrast, platelet antibodies against type I alloantigens seem to be restricted to PTR. (Santoso et al., 2003)

Blood group antigens on platelets include ABH, Lewis, Ii and P antigens. These antigens are present in platelet membrane glycoproteins (Santoso S et al, 1991). Antibodies to ABO, Lewis, and P antigens often develop spontaneously in antigen-negative persons, and antibodies to I antigens are cold reacting autoantibodies (Mollison PL et al, 1993). It is generally assumed that the expression of ABH antigens on platelets is too weak that anti-A, anti-B isoagglutinins cannot affect considerably the survival time of ABO-incompatible platelets (Santoso et al, 2003) and although the impact of ABO compatibility on post-transfusion platelet recovery, albeit small, has been demonstrated (Lee EJ et al., 1989).

Platelet membrane glycoproteins contain antigenic epitopes that are not found on other types of blood cells. More than 20 such platelet specific alloantigens have been identified (Kunicki et al, 1992), and many have been well characterized at the molecular level (Newman et al, 1995). Many platelet-specific alloantigens have been found on other cells and tissues as well such as the cell adhesion receptor and intergrins. Six diallelic alloantigen systems (HPA-1-5 and HPA-15) and a number of low frequency antigens (HPA-6W-14W, HPA-16W) have been described. Currently, six platelet membrane glycoproteins GPIa, GPIbα , GPIbß , GPIIb, GPIIIa and GPI-linked CD109 have been identified as carriers of platelet alloantigenic determinants (Santoso et al., 2003). These platelet specific alloantigens are present in dimorphic forms, and the allelic frequencies are equally distributed in a high-frequency public form and a low-frequency private form (Kim et al., 1995). They can elicit the production of antibodies through transfusion and pregnancy. The development of such antibodies can result in three clinical conditions:

1. Neonatal alloimmune thrombocytopenia- mother becomes immunized to platelets of fetus and give birth to thrombocytopenic infants;

2. Post-transfusion purpura- patients become immunized to platelet-specific alloantigens after blood transfusion and develop thrombocytopenia;

3. Transfusion-associated alloimmune thrombocytopenia- patients receive multiple platelet transfusions and become refractory to random-donor platelets. (Mcfarland, 1996)

Table 1.4. Human Platelet-Specific Antigen Systems (Adapted from Kim et al, 1995)

Platelet Antigen System	Protein Antigen	Synonyms	Alleles	Antigen Frequency
HPA-1	GPIIIa	PI^A,Zw	HPA-1a = PI^{A1} HPA-1b = PI^{A2}	97% 26%
HPA-2	GPIb	Ko, Sib	HPA-2a HPA-2b	99% 14%
HPA-3	GPIIb	Bak, Lek	HPA-3a HPA-3b	85% 66%
HPA-4	GPIIa	Pen, Yuk	HPA-4a HPA-4b	>99% <1%
HPA-5	GPIa	Br, Hc, Zav	HPA-5a HPA-5b	99% 20%

The platelet membrane glycoproteins (GPs) that carry the human platelet antigen (HPA) epitopes play a fundamental role in platelet function so that platelet alloantibodies may not only cause thrombocytopenia, but may also affect primary haemostasis. (Hoffbrand et al., 1999)

Moreover, HPA antibodies have been found in approximately 20% of alloimmunized patients. (Kiefel et al., 2001)

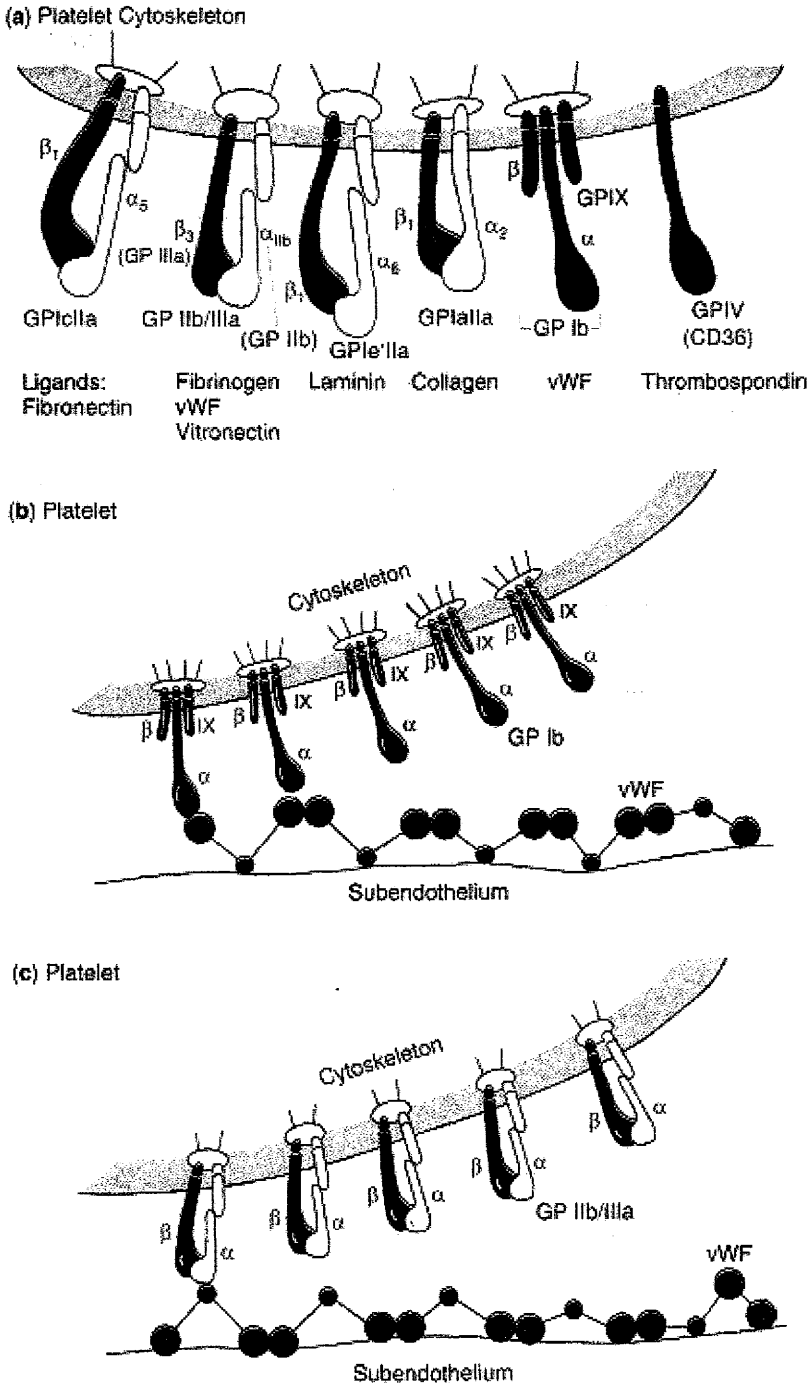

Figure 1.1 Platelet membrane glycoproteins and adhesion to membrane (Adapted from Hoffbrand et al.,1999)

The HLA class 1 molecules constitute the third group of platelet alloantigens. These antigens are heterodimeric membrane glycoproteins that consist of a 44-kD highly polymorphic heavy chain and a 12-kD invariant $ß_2$-microglobulin. These two polypeptides are noncovalently associated with each other and are present in varying quantities on most cells in an individual. The genes encoding HLA heavy chains are located at three different loci (A, B and C) of chromosome 6. On platelets, the expression of HLA-A and B antigens is higher than that of HLA-C antigens. (Santoso et al, 2003)

Functionally, HLAs play a major role in presenting antigenic peptides to cytotoxic T lymphocytes (CTLs) (Zinkernagel et al, 1979) and are essential for the ontogenetic development of CD8+ cytotoxic T cells in the thymus. The expression of HLA antigens on platelets can vary substantially and is influenced by gene dosage and other as yet uncharacterised genetic factors (Liebert et al, 1979) Antibodies to HLAs are responsible for greater than 90% of immune-mediated platelet transfusion refractoriness. (Mcfarland, 1996).

The most common platelet reactive antibodies in transfused patients recognize epitopes on HLA class I molecules which are frequently responsible for febrile nonhemolytic transfusion reactions and immunologically mediated PTR. Although most cases of antibody-mediated PTR related to HLA class I, additional platelet specific antibodies have been identified in sera of transfused patients.

Risk factors for developing HLA antibodies include the presence of more than 1 million donor leucocytes in transfused products, transfusing ABO-mismatched platelets, the presence of an intact immune system (ie absence of cytotoxic or immunosuppressive therapy), female sex (approximately 75% of cases), and a history of multiple transfusions (>20). (Sepulveda et al., 2001)

Refractoriness to platelet transfusion that accompanies alloimmunization is one of the most serious and most difficult to manage among transfusion therapy hazards. It occurs when a patient receives platelet preparation from another person that contains leucocytes. The body reacts to the leucocytes and develops antibodies against them (HLA antibodies). This immune response, called alloimmunization, can cause patients to become refractory (resistant) to subsequent platelet transfusions. This means that they do not respond or benefit from the transfusion, whereby the risk of spontaneous bleeding from the patient. Patients receiving leucocyte-reduced blood products are at much lower risk for refractoriness to platelet transfusion than the recipients of blood that is not leucocyte reduced. (Kruskall, 1997)

There are clinical syndromes associated with platelet alloimmunization:

1. Human leucocyte antigens: alloimmunization causes refractoriness to platelet transfusion.This can be reduced by the use of leucocyte-depleted blood components. There are few reported cases of refractoriness due to HPA alloimmunization.

2. Human platelet antigen alloimmunization causes NAIT and PTP. Most HPAs have been implicated in NAIT. Furthermore, HPAs which is associated on GPIIb-IIIa are primarily, if not exclusively, involved in PTP, and the immunochemical properties of this complex may be related to the mechanism of platelet destruction in PTP. (Hoffbrand et al., 1999)

Patients not previously sensitized to develop anti-platelet antibodies approximately 3-4 weeks (10 days to 26 days) after the transfusion and patients previously immunized by transfusion, pregnancy, or organ transplantation, anamnestic responses occur as early as 4 days after transfusion. Macrophages in the liver, spleen, and other tissues of the mononuclear phagocyte system phagocytize and destroy antibody-coated platelets. (Sepulveda et al., 2001)

Other than alloimmunization to platelet antigens, there is also alloimmunization to red cells and granulocytes in multiply transfused patients. (Sepulveda et al., 2001)

Approximately 20-85% of patients who receive multiple transfusions become immunized against platelet antigens (eg HLA, HPA) and approximately 30% of patients who are alloimmunized develop refractoriness to platelet transfusions (Sepulveda et al., 2001). Refractoriness may be caused by immune causes as well whereby the antibodies present in the patient's serum, which can react with antigens present on the platelet membrane and decrease platelet in vivo survival and function. (Rebulla, 2002). In approximately 66% of these patients, non immune factors such as sepsis, antibiotics (for example

amphotericin), hypersplenism, fever, active bleeding and DIC alone are the cause, whereas alloimmunization may be involved in 33% of refractory patients, often in combination with nonimmune causes. (Sepulveda et al., 2001)

Refractoriness to platelet transfusion is most commonly seen in patients with haematologic diseases requiring frequent red blood cell and platelet transfusions. Platelet refractoriness is not frequently seen in surgical patients, who may require less prolonged and intense platelet support than haematology and oncology recipients. (Kaneda et al., 1999)

Frequently, patients with refractoriness to platelet transfusion are asymptomatic and diagnosed by laboratory methods; however, failure to achieve hemostatic levels of platelets may preclude these patients from important procedures, including bone marrow transplantation. Alloimmunization should be avoided at all costs in candidates for bone marrow transplantation. In platelet refractoriness patients, preexisting bleeding resulting from thrombocytopenia may persist after transfusion of an appropriate therapeutic dose of platelets and rarely, spontaneous bleeding may occur after prophylactic transfusion of platelets. (Sepulveda et al., 2001)

But it should be remembered that platelet transfusions in a bleeding patients are usually associated with very low post-transfusion platelet count increments. Nonetheless, such transfusions are considered a success if they are associated with cessation of bleeding. (Rebulla, 2002)

In general, alloimmunization results in the rapid removal of platelets and in lower counts at 10 minutes to 1 hour post-transfusion, whereas nonimmune causes mostly affect the 4- to 24-hour post-transfusion count. Mild alloimmunization, however, can be present with 1-hour increments within the reference range. (Sepulveda et al., 2001)

Strategies to overcome platelet transfusion refractoriness include the selection of platelets from HLA-identical or HLA-compatible donors from HLA-typed donor registries, (Yankee et al, 1969), platelet cross-matching (Murphy et al, 1998), and the antibody specificity prediction method (Petz et al., 2000).

1.6. The immune response

This immune response can be due to human platelet antigens and human leucocyte antigens.

1.6.1. Immune response to Human platelet antigens

An immune response to HPAs is an exception rather than a rule. The most frequently occurring antibody is anti-HPA-1a which is found in 80-90% of cases. Anti HPA-5b occurs in 10-15% of the cases, and there are only occasional detections of antibodies to the other HPAs. These antibodies almost always occurs in women who have been immunized by pregnancy or in association with PTP. The increased use of platelet transfusion since the early 1980s has not altered this situation; there are few reported cases of refractoriness due to platelet specific alloantibodies, and the incidence and specificity of these alloantibodies in this situation is still uncertain. Although anti-HPA-1a is the most frequently encountered antibody, less than 10% of HPA-1a-negative individuals who are exposed to HPA-1a become immunized. (Hoffbrand et al., 1999)

1.6.2. Immune response to Human leucocyte antigens

Alloimmunization to HLA associated with platelet transfusions is caused by the passenger leucocytes especially the donor dendritic cells, which directly activate the recipients helper T-lymphocytes. Platelets alone seem to be unable to induce primary antibody responses against HLA antigens, due to the absence of HLA class II antigens. (Hoffbrand et al, 1999)

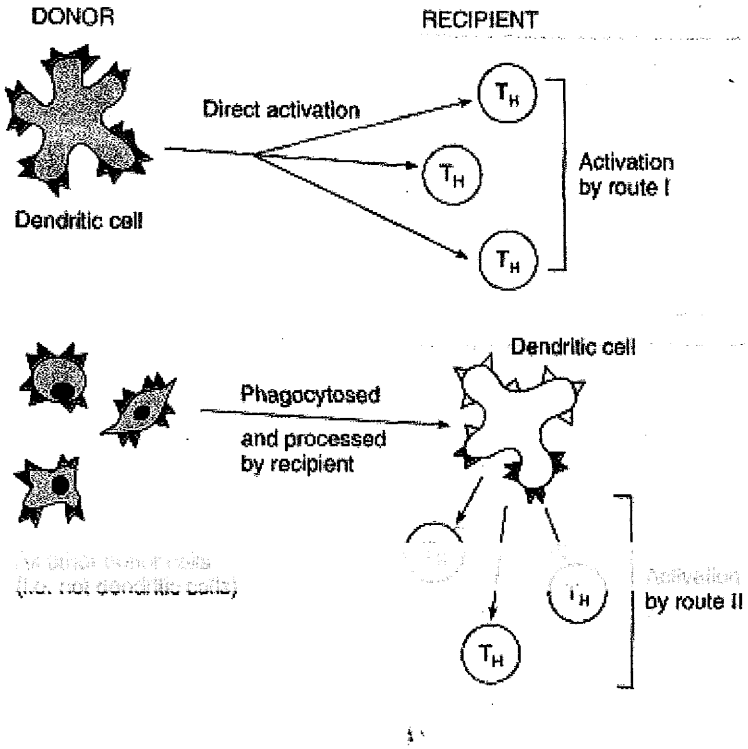

Figure 1.2 Dual route of sensitization by allo-antigen. (Adapted from Hoffbrand et al.,1999)

1.7. Laboratory detection of platelet alloantibody.

1.7.1 Enzyme Linked Immuno-Sorbant Assay (ELISA)

The basic principle of ELISA is to use an enzyme to detect the binding of antigen (Ab) and antibody (Ab). The enzyme converts a colourless substrate (chromogen) to a coloured product, indicating the presence of antigen:antibody binding. An ELISA can be used to detect either the presence of antibodies or antigens in a sample, depending on how the test is designed.

If the test is designed to detect Abs in a sample:

1. Combine Ag and Ab; allow them to bind:

- the antigen is firmly attached to a surface (usually plastic well or filter)
- sample being tested is added , allowed to incubate , then unbound Abs are washed from the surface.

2. Detect Ag:Ab binding:

- an antiglobulin that is covalently attached to an enzyme (ie enzyme-linked) is added, incubated, then unbound antiglobulins are washed from the well.
- a colourless substrate of the enzyme is added
- if the enzyme-linked antiglobulin bound to Abs on the surface, the

enzyme will convert the colourless substrate to a coloured product.

If the test is designed to detect Ags in a sample:

1. Combine Ag and Ab; allow them to bind:

   - an Ab that reacts with the Ag is firmly attached to a surface

   - sample being tested is added , allowed to incubate

2. Detect Ag:Ab binding

   - a second Ab that reacts with the Ag and that is covalently attached to an enzyme (ie enzyme-linked) is added allowed to incubate, then unbound enzyme-linked Abs are washed from the surface

   - a colourless substrate of the enzyme is added

   - if the enzyme-linked Ab bound to Ags on the surface, the enzyme will convert the colourless substrate to a coloured product. (Smith et al, 2003)

There are a few components of ELISA:

1. Solid phase

- consists of microtitre plates, strips or polystyrene beads coated with the appropriate Ab or Ag.

2. Conjugate

-An appropriate enzyme-labelled ligand (usually an antibody). This Ab can be specific for an organism or an Ag of interest , or can be directed against species – specific Ab class eg human IgM.

- Organism-specific conjugates are used in SANDWICH, COMPETITIVE and CAPTURE assays. Ab class-specific conjugates are used in INDIRECT assays. (Collins, 1995)

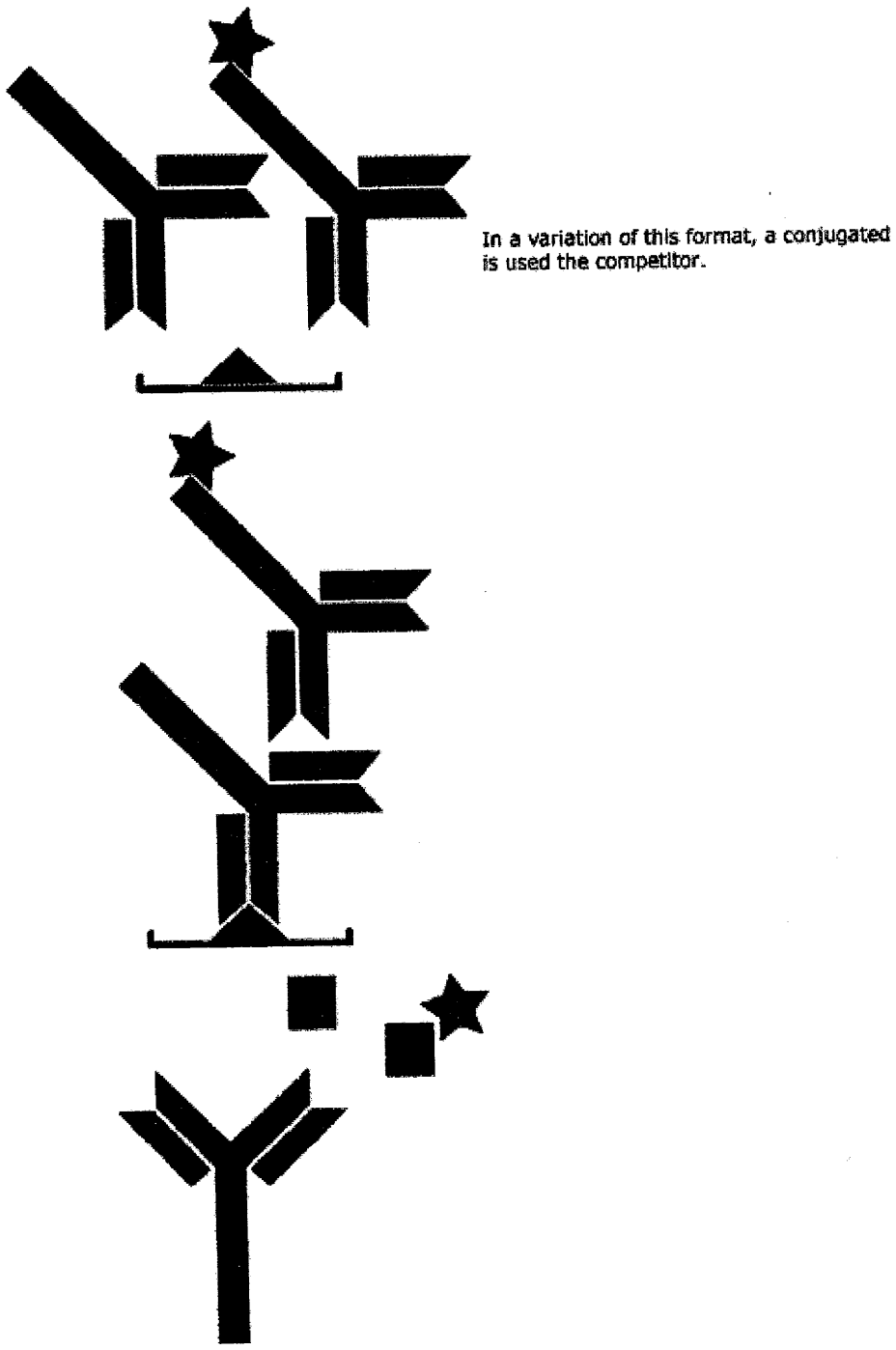

Figure 1.3 Coating antigen/antibody to the solid phase (Adapted from Collins, 1995)

In the past two decades considerable progress has been made in the characterization of platelet alloantigens. The use of serological antigen capture assays such as the monoclonal antibody immobilization of platelet alloantigens (Kiefel et al, 1987) and immunochemical methods led to rapid increase in the number of newly recognized platelet alloantigens. (Santoso et al., 2003)

1.7.2 Other Methods

Many methods have been described to identify platelet antibody such as:

1. lymphocytotoxicity (LCT)
2. platelet adhesion immonoflourescence test (PAIFT)
3. monoclonal antibody immobilization of platelet antigens (MAIPA)
4. solid phase capture platelet test (CPT).

The sensitivity, specificity and reproducibility of the solid phase capture-platelet test (CPT) has been compared with the other tests. The CPT has a higher sensitivity than PAIFT ($P<0.001$) and LCT ($P<0.01$). The specificityof CPT was 86%, which is lower than LCT (100%) ($P<0.01$). Both the CPT test and PAIFT were comparable with the regard to reproducibility (100% versus 92%). The solid phase assay is easier to handle than PAIFT and LCT, allowing identification of HLA as well as of platelet specific antibodies. (Santoso et al., 2003)

Chapter 2

AIMS AND OBJECTIVES

2.0 AIMS AND OBJECTIVES

2.1. The aim of this study was to determine the prevalence and the risk factors of platelet alloantibodies in patients receiving multiple transfusions.

2.2. Specific objectives

a) To identify the spectrum of platelet specific antibodies that is detected in multiply transfused thrombocytopenic patients.

b) To identify the outcome of platelet transfusion in alloimmunized patients in terms of platelet count and symptoms (such as mucosal bleed, petechiae).

c) To identify the risk factors to platelet alloantibodies development (such as sex, age and underlying disease).

Chapter 3

MATERIALS AND METHODS

3.0. MATERIALS AND METHODS

3.1. Study design

This was a cross sectional study conducted at Hospital Universiti Sains Malaysia from June 2003 to June 2004. This study was approved by the School of Medical Sciences Research and Ethical Committee. All subjects gave an informed written consent (Appendix 1).

3.2. Sample size calculation

The primary end-point of this study is to look for the presence of platelet alloantibodies in multiply transfused thrombocytopenic patients. The values were based on a previous study, whereby 9.6% of patients had platelet specific antibodies (Uhrynowska et al., 1996).

Using statistical analysis for sample size: single proportion

$$N = \frac{Z\,p(1-p)}{\Delta}$$

where;

N= number of subject

Z=1.96

Δ=0.05

p=9.6%

$N = (1.96^2)/0.05 \times 0.096 \times (0.904)$

$= 134$ sample

3.3. Selection of patients and study protocol

3.3.1. Patient

Subjects were recruited from wards, medical clinic and transfusion records from blood bank. Subjects with history of multiple transfusions (more than twice of any of blood components, more than 2 weeks but not more than 2 years of their last transfusions) and thrombocytopenia (platelet count less than 100×10^9/l). Patients who were on heparin or with autoimmune diseases such as ITP, SLE or AIHA were excluded. Selected patients were then given written consent. 7 mls of peripheral blood were taken and collected in EDTA bottles. Two mls of the peripheral blood for FBP and 5 mls for antibody detection. The analysis was performed at the Haematology Laboratory, HUSM.

3.3.2. Laboratory tests:

The laboratory tests performed were:

1. Full blood picture
2. Screening test for platelet antibodies.

3. Confirmation test.

Full blood picture was done as a screening to confirm thrombocytopenia. Sysmex NE-8000 machine was used for full blood count and haemogram. Technology employed in automated differential count was impedance with low-frequency direct count. Thin blood films were prepared and dried in the air. Each film was left for 3 minutes in the fixative. The slides were then left in the diluted staining solution (Wright stain) for 10 minutes, which the slides were rinsed with phosphate buffered solution, pH 5.8, for 1 minute. Lastly the slide was rinsed with water, air dried and mounted.

The remaining 5 mls of blood was centrifuged for 5 minutes at 1000 rpm to harvest the platelet rich plasma (Figure 3.1). Once the plasma was separated and stored at -70°C before the screening was done. Screening was performed by using Capture –P Solid phase system from Immunocor Inc. (Figure 3.2). Donor platelets were used as a source of platelets. Samples of donor platelets were collected into EDTA, CPD, or CPDA-1 (clotted specimens cannot be used). Platelet-rich plasma was separated from red cells immediately after collection.The first step of the Capture-P procedure was to prepare platelet monolayer by adding donor platelet group O into the test wells that coated with anti-platelet antibodies. Antibodies act as coupling agents to keep the platelets firmly attached to the surfaces of the plastic wells throughout the testing procedure. The platelets were attached to the coupling agent by the forces placed on them by centrifugation. Excess unbound platelets and plasma, were decanted and the strip washed six with isotonic solution. Immediately 2 drops (100 ± 10µL) of Capture LISS were added to the wells containing platelets. One drop (50 ± 5µL) of the control serum (strong

positive, negative and weak positive) were added into the control wells. In the remaining wells one drop (50 ± 5µL) of the patient's platelet rich plasma was added. The plate was gently agitated to mix the sera and incubated at 36-38°C for 30-60 minutes. The wells then were washed 6 times with isotonic solution. Immediately 1 drop (50 ± 5 µL) of Capture-P Indicator red cells (containing anti-IgG) was added. The plate then was centrifuged to bring the indicator red cells in contact with antibodies bound to the immobilized platelets. The results were compared with those obtained with the control sera. A positive case was characterized by adherence of the indicator red cells spread over the surface of the well bottom and a negative reaction was indicated by a tight button in the center of the well bottom. (Figure 3.3). The screening test was done in batches whereby for 1 kit, 42 tests can be done (1 kit contains 48 wells, 6 wells for control sera and the remaining 42 wells for the test samples).

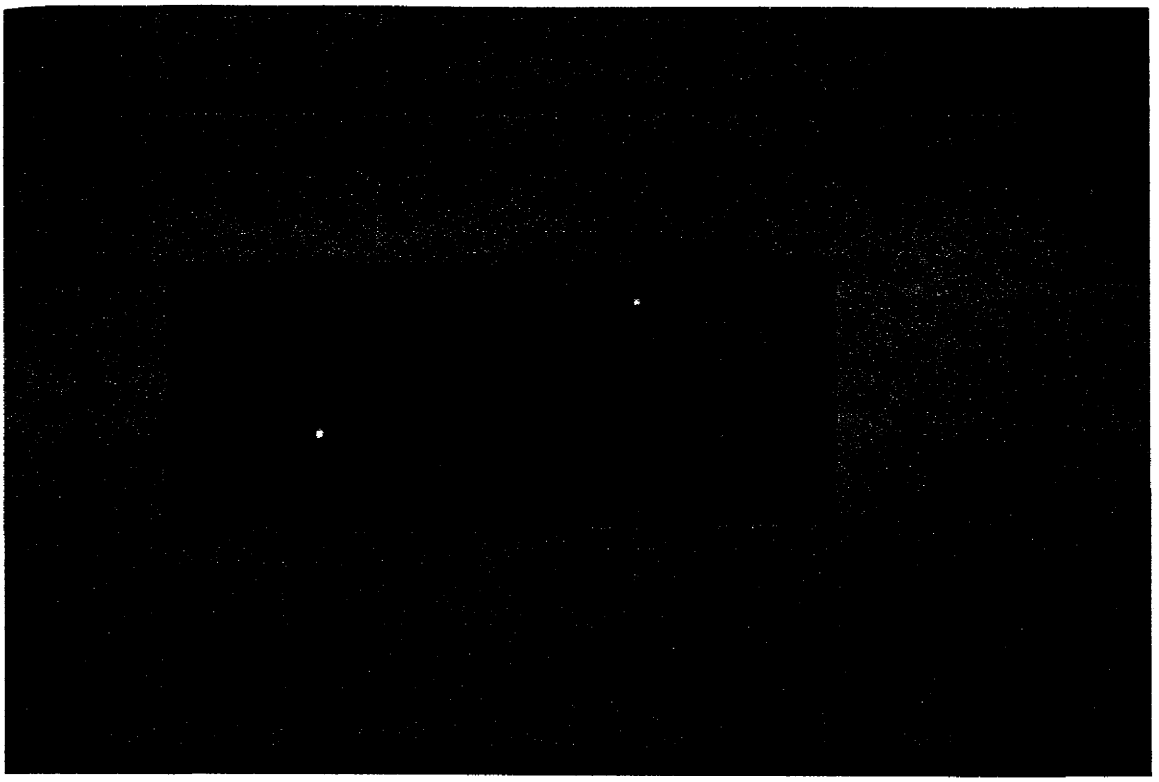

Figure 3.1 The centrifuge machine (PK 130R)

Figure 3.2 Capture-P for screening test.

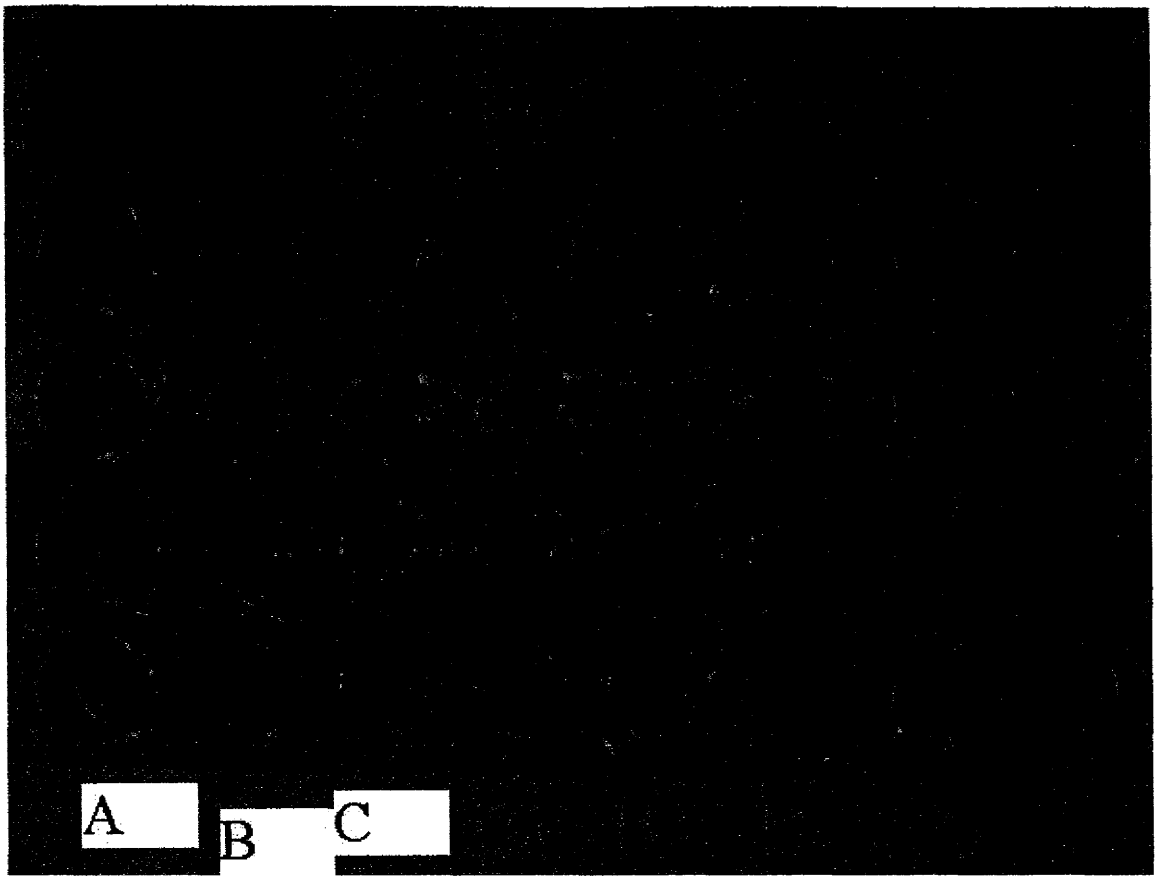

Figure 3.3 Results of Capture-P screening test.(A= strong positive, B= negative, C=weak positive)

Positive cases, were confirmed by the Capture-P Ready Screen test (Figure 3.5) the strips of which contained 16 wells, 4 for control well and another 12 for the positive screening sample. These 12 wells have been coated with platelets in monolayer. Platelets from twelve different donors have been used to coat twelve wells on each strip. Two drops (100 ± 10μL) of Capture LISS was added into all wells except the blank well (1 out of those 4 wells). One drop (50 ± 5μL) of control sera were then added into the designated

wells (containing a pool of platelets). One drop (50 ± 5µL) of patients plasma samples were added into the other 12 wells. The strip was then mixed and incubated at 36-38°C for a minimum of 30 minutes. The strip was washed 6-8 times to remove unbound immunoglobulins. Immediately 1 drop (50 ± 5µL) of Capture-P Indicator red cells were dropped into each wells. The strip was centrifuged and examined for the presence or absence of Indicator red cells adherence. For the test results to be considered valid, the following reactions was obtained with the Capture-P Control sera each time a plate was tested. Positive test was characterized by adherence of Indicator Red Cells over the entire reaction surface forming an even thin red cell layer , and for the negative test a button of Indicator Red Cells at the bottom of the test wells with no readily detectable area of adherence. (Figure 3.4)

Figure 3.4 Results of Capture-P confirmation test

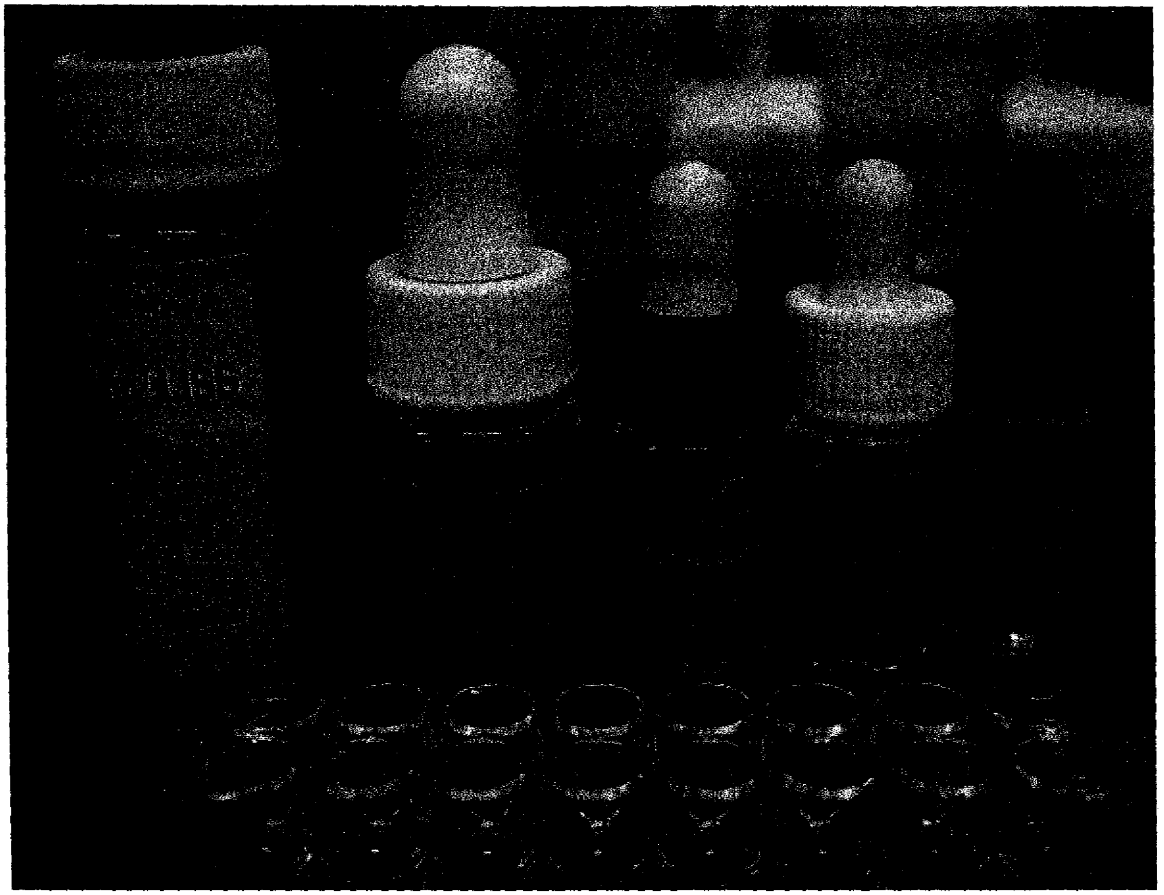

Figure 3.5 Capture-P confirmation test.

3.4. Statistical analysis

The data was analysed by using SPSS software, Descriptive analysis and Chi-square (to associate the presence of antibody with age, diagnosis, number of plasma and packed cells transfused).

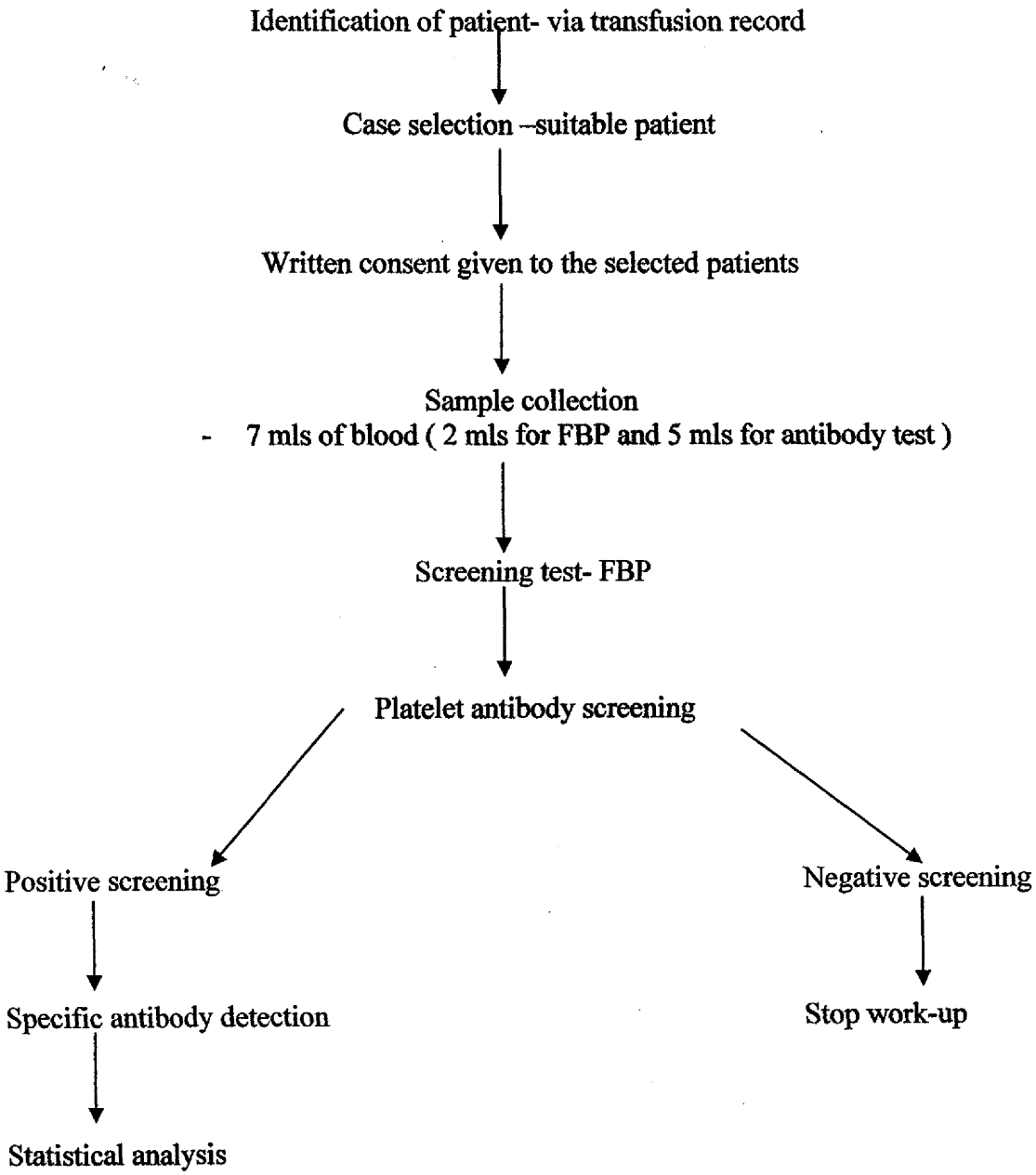

Figure 3.6 Flow chart of the study protocol.

Chapter 4

RESULTS

4.0 RESULTS

A prospective study was performed at the Haematology Laboratory, Hospital Universiti Sains Malaysia from June 2003 to June 2004. 95 patients were recruited from Hospital USM who were thrombocytopenic and received multiple blood transfusion.

4.1 Demographic data

Of the 95 patients in this study, 63 patients (66.3%) had haematological disorders (Acute Lymphoblastic Leukaemia, Acute Myeloid Leukaemia, Non-Hodgkin Lymphoma, Hodgkin's Disease, Chronic Myeloid Leukaemia, Chronic Lymphocytic Leukaemia, Aplastic Anaemia and Myelodysplastic Syndrome) and 32 patients (33.7%) had non-haematological disorders (Chronic liver disease, End-stage renal failure and solid tumours). The history of the diseases was based on data collected from patient's record. Fifty patients (52.6%) were female and 45 patients (47.4%) were males. There were 88 Malays (92.6%), 5 Chinese (5.3%), 1 Indian (1.1%) and another 1 was Siamese (1.1 %). The age of the patients ranged from 3 years to 90 years with the mean age of 40.3 years old (Table 4.1b). Most of the patients in this study were more than 40 years old. The numbers of packed cell that have been transfused were between 1 unit to 29 units with a mean of 8.8 units throughout the course of this study. The numbers of platelet concentrate that have been transfused were between 1 unit to 168 units with a mean of 25.43 units throughout the course of this study (Table 4.1a & b).

Table 4.1a Demographic data of patients with multiple transfused thrombocytopenia.

Demographic data	Number of patients (n)	(%)
Total Patients	95	
Diagnosis		
Haematological disorders	63	66.3%
Non-Haematological disorders	7	7.4%
-Chronic Liver Disease	11	11.6%
-ESRD	3	3.2%
-Others	11	11.6%
Gender		
Female	50	52.6%
Male	45	47.4%
Ethnic		
Malay	88	92.6%
Chinese	5	5.3%
Indian	1	1.1%
Others	1	1.1%
Age		
0-20 years	28	29.5%
21-40 years	15	15.8%
41-60 years	32	33.7%
61-80 years	17	17.9%
>80 years	3	3.2%

Table 4.1b Demographic data of thrombocytopenic patients who received blood transfusion

Demographic data	Number (n)	%	Mean ± SD
Age of patients			40.3 ± 24.1
0-20 years old	28	29.5%	
21-40 years old	15	15.8%	
41-60 years old	32	33.7%	
61-80 years old	17	17.9%	
>80 years old	3	3.2%	
Number of packed cells transfused (units)			8.8 ± 6.6
1-10	52	54.7%	
11-20	29	30.5%	
>20	6	6.3%	
Nil	8	8.4%	
Number of platelet transfused (units)			25.4 ± 28.5
1-10	25	26.3%	
11-20	22	23.2%	
>20	41	43.1%	
Nil	7	7.4%	

4.2 Data of patients who developed platelet antibodies

Platelet alloantibodies were detected in 7 (7.4%) patients. Six of them were diagnosed to have haematological disorders (3 were diagnosed to have Aplastic Anaemia, 1 with AML, 1 with NHL and another 1 with Familial thrombocytopenia). Another 1 patient was involved in Motor Vehicle Accident. The age of the patients were between 12 years old to 75 years old. There were 2 males and 5 females. All of them were Malays. The number of packed cells that had been transfused was between 1 unit to 25 units. The number of platelet concentrate that had been transfused was between 1 unit to 139 units. Four (4.2%) patients were found to have anti-HPA 5b and another 3 (3.2%) patients were found to have non-specific pattern (Table 4.1c).

Table 4.1c Data of patients with platelet alloantibodies

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age	75	46	12	17	50	50	39
Sex	Male	Female	Female	Male	Female	Female	Female
Race	Malay	Malay	Malay	Malay	Malay	Malay	Malay
Diagnosis	NHL	Aplastic anaemia	Familial thrombocy-topenia	MVA	Aplastic anaemia	Aplastic anaemia	AML
Number of packed cells transfused	25	0	11	10	6	5	7
Number of platelet transfused	39	8	25	16	0	24	139
Type of alloantibody	Non specific pattern	HPA-5b	HPA-5b	HPA-5b	No specific pattern	No specific pattern	HPA-5b

Table 4.1d Outcome of platelet transfusion to alloimmunized patients

	Outcome
Patient 1	Pre transfusion platelet count between 30 to 41 x 10^9/l. Post transfusion platelet count between 60 to 80 x 10^9/l with infrequent bruising.
Patient 2	Pre transfusion platelet count between 10 to 29 x 10^9/l. Post transfusion platelet count between 90 to 142 x 10^9/l with no bleeding tendencies.
Patient 3	Pre transfusion platelet count between 20 to 30 x 10^9/l. Post transfusion platelet count between 32 to 148 x 10^9/l with infrequent bleeding tendencies.
Patient 4	Pre transfusion platelet count between 20 to 33 x 10^9/l. Post-transfusion platelet count between 54 to 73 x 10^9/l during admission in neurosurgical ward. Patient lost from follow up for neurosurgery (? Absconded).
Patient 5	Pre transfusion platelet count between 25 to 29 x 10^9/l. Post transfusion platelet count between 64 to 90 x 10^9/l.
Patient 6	Pre transfusion platelet count between 4 to 10 x 10^9/l. Post transfusion platelet count between 20 to 51 x 10^9/l with occasional bleeding tendencies.
Patient 7	Pre transfusion platelet count between 1 to 4 x 10^9/l with post transfusion platelet count between 4 to 7 x 10^9/l. Died due to septicaemia.

Table 4.1e Association between presence of platelet alloantibody with age, sex, race, number of packed cells and plasma transfusion.

Independant	Absence of alloantibody	Presence of alloantibody	Statistical Tests	P value
Age				
0-40	40	3	0.06	0.807
>40	48	4		
Gender				
Female	45	5	1.19	0.275
Male	43	2		
Ethnic				
Malay	81	7	0.00	0.999
Non Malay	7	0		
Number of packed cells transfused			0.87	0.350
<20	83	6		
>20	5	1		
Number of platelet transfused			0.008	0.927
<20	50	4		
>20	38	3		

(Level of significance, $p < 0.05$)

4.3 Association between presence of platelet alloantibody with age, sex, race, number of packed cells and plasma transfusion.

There was no significance relationship between presence of platelet alloantibody and age of patient (P value > 0.05). This is probably due to small number of patient recruited in this study.

There was also no significant relationship between presence of platelet alloantibody with sex and race of the patients. However all patients with platelet alloantibody were Malays and 2 of them were males and the rest were females.

The number of packed cells and platelet concentrate transfused also showed that it was not associated with the presence of platelet alloantibody. (Table 4.1e).

Chapter 5

DISCUSSIONS AND LIMITATIONS

5.0 DISCUSSIONS AND LIMITATIONS

Alloimmunization in polytransfused patients is a well known observation which very often results in refractoriness to platelet transfusions (Uhrynowska et al., 1996). As mentioned earlier, alloantibodies are usually directed against HLA antigens and their frequency is evaluated between 20% to 70% (Slichter et al., 1994). Alloantibodies may also be directed against platelet-specific antigens but their frequency is controversial and rare (2.1%) (Legler et al., 1997).

Legler *et al.*, 1997 has shown that refractoriness to platelet transfusions was due to alloimmunization in 17.5% of patients and due to non-haematological causes (ie fever and sepsis) was around 62.5%.

The purpose of this prospective study was to determine the frequency and specificity of platelet alloantibody in these multiply transfused patients and also to correlate with any underlying disease, age of patients, sex of patients, race of patients, and also the numbers and types of blood components given to the patients with the development of platelet antibodies.

The majority of the patients were diagnosed as haematological disorders (66.3%) and the remaining 33.7% were diagnosed to have non-haematological disorders (end-stage renal failure, chronic liver disease and others). In these non-haematological patients, most of them were having fever and sepsis during platelet alloantibody tests was undertaken.

5.1 Type of platelet alloantibody

In this study, seven (7.4%) of the patients were found to have platelet alloantibody. The anti-HPA-5b was found to be positive in 4 (4.2%) of these patients and 3 (3.1%) of them were female and only 1 (1.1%) was male. Another 3.2% of patients were shown to have non-specific pattern of platelet alloantibody.

The finding of anti-HPA-5b as the highest frequency in this study was similar with the finding by Kiefel *et al.*, 2001 which had shown that the antibody specificity found with the highest frequency was anti-HPA-5b (Br^a). Other specificities found (in decreasing order of frequency) were anti-HPA-1b (Pl^{A2}), anti-HPA-5a (Br^b), anti-HPA-2b (Ko^a), and anti-HPA-1a (Pl^{A1}).

Reports about frequency and specificity of platelet-specific alloantibodies in multitransfused patients are rare. This is due to the fact that in such patients platelet-specific antibodies will occur rarely without HLA antibodies, thus complicating their identification and especially their specificities (Murphy et al., 1990).The possibility of having the non-specific pattern of platelet alloantibodies in this study (3.2%) may be due to the presence of anti-HLA especially anti-HLA 1 which is the commonest to be presence in leukaemia patients.

As mentioned before, Capture-P test was used in this study. And Kiefel *et al.*, 2001 used Platelet Adhesion Immunoflourescence Test (PAIFT) for the typing of platelet antibodies.

Jin *et al.*, 1993 has shown that the Capture-P test has a higher sensitivity than PAIFT ($p<0.001$) and the specificity of the Capture-P was almost similar with PAIFT (86%).

Kiefel *et al.*, 2001 demonstrated that the prevalence of platelet-specific antibodies in all patients was 20 (8%) of 252 patients; in 5 (2%) of 252 patients, platelet-specific alloantibodies (anti-HPA-5b in 4 patients and anti-HPA-1a in 1) were not accompanied by HLA class 1 specific antibodies. Of the 108 patients with HLA antibodies, 15(13.9%) exhibited additional platelet-specific alloantibodies. Anti-HPA-5b was found almost exclusively in female patients: only 1 of 10 examples of anti-HPA-5b was found in a male patient. In 10 patients (4%), they identified platelet-specific antibodies with "broad" specificities (i.e., the antibodies reacted with one or more platelet GPs and usually with all platelets of the test panel). Such antibodies have been observed by other authors and were referred to in the literature as "autoantibodies" or "panreactive" antibodies. Panreactive antibodies were observed by Kurz *et al.*, (1996) in 26 percent of patients, while, in 20 percent, they were associated with HLA antibodies. In studies published by Novotny *et al.*, (1995) and Godeau *et al.*, (1992), most antibodies with undefined specificity were not associated with refractoriness to transfused platelets.

As the study done by Tanaka *et al.*, (1996), anti-HPA-5b was found to be the commonest platelet-specific alloantibody in Korean and Japanese population where the alleles of the human platelet antigen (HPA) was 0.02 (Table 5.1), and this supports the finding of having anti-HPA-5b as the commonest platelet alloantibody in our population.

Table 5.1 Allele frequencies of the HPA-1 to HPA-6 systems in different populations. (Adapted from Ferrer et al., 2002).

	HPA-1 alleles		HPA-2 alleles		HPA-3 alleles		HPA-4 alleles		HPA-5 alleles		HPA-6 alleles		
Populations	1a	1b	2a	2b	3a	3b	4a	4b	5a	5b	6a	6b	Reference
Berber	0·75	0·25	0·82	0·18	0·68	0·32	1·0	0·0	0·86	0·14	1·0	0·0	This study
Spanish	0·81	0·19	0·90	0·10	0·65	0·35	1·0	0·0	0·88	0·12	1·0	0·0	Muñiz-Diaz *et al.* (1998)
Austrian	0·85	0·15	0·92	0·08	0·61	0·39	–	–	0·89	0·11	–	–	Holensteiner *et al.* (1995)
Dutch	0·85	0·15	0·93	0·07	0·55	0·45	1·0	0·0	0·90	0·10	–	–	Simsek *et al.* (1993)
Finns	0·86	0·14	0·91	0·09	0·59	0·41	–	–	0·95	0·05	–	–	Kekomaki *et al.* (1995)
Korean	0·99	0·01	0·92	0·08	0·55	0·45	0·99	0·01	0·98	0·02	0·98	0·02	Seo *et al.* (1998)
Japanese	0·99	0·01	–	–	–	–	0·99	0·01	–	–	0·97	0·03	Tanaka *et al.* (1996)

In the United States, HLA class 1 antibodies were involved in majority of the alloimmunized cases, whereas platelet specific antigens (e.g. HPA) were involved in approximately 10-20% of refractory cases. Both types of antibodies were involved in approximately 5% of cases. A single random RBC or platelet transfusion induces anti-HLA antibodies in fewer than 10% of recipients (most likely related to the tolerogenic effect of blood transfusions). If patients have more than 20 transfusions, they become sensitized in increasing proportions; after 50 transfusions, the majority (as many as 70%) of patients have anti-HLA antibodies. The presence of HLA antibodies showed better correlation with platelet refractoriness than antibodies directed against platelet-specific

antigens. The most common platelet-specific antibody was directed against the HPA-1a antigen present on glycoprotein 11a (Sepulveda et al., 2001).

Refractoriness to platelet transfusions due to platelet-specific antibodies is often difficult to assess since, if they developed anti-HLA antibodies, such patients must be transfused with HLA-matched platelets. Nevertheless, platelet-specific antibodies (HPA-1a, HPA-1b, HPA-2b, HPA-3a) have already been found to be responsible for platelet refractoriness (Kicklert et al., 1990). Study done by Uhrynowska *et al.*, (1996), showed that the efficacy of platelet transfusions was difficult to assess due to other non haematological factors which could contribute to refractoriness (Doughty et al., 1994).

Alloantibody specificities encountered in multiply transfused patients differ considerably from those in patients with PTP and maternal alloantibodies in NAIT. Two latters conditions, anti-HPA-1a was the platelet antigen, most frequently encountered among white patients Kroll *et al.*, (1998), whereas anti-HPA-1b and anti-HPA-5b were prevalent among multiply transfused patients. In a large series of multiparous blood donor, anti-HPA-5b was the most common alloantibody (Kalinowski et al., 1997). However, anti-HPA-1a was the second most frequent alloantibody in that donor group, which reflects the different mode of immunization in that study population (pregnancy).

The frequency of platelet-specific antibodies in multitransfused patients and their implication in platelet transfusion refractoriness has been a matter of controversy for years. The main reasons are the difficulties encountered in differentiating between HLA- and platelet-specific antibodies and, within platelet-specific antibodies, between auto- or

alloantibodies, using the available platelet antiglobulin test (Schnaidt et al, 1996). Thus, data suggesting a frequency of platelet-specific antibodies in about 20% of patient were obtained indirectly, such as different reaction patterns of sera tested on lymphocytes and platelets of the same individual as well as transfusion failures with HLA-matched platelets (Pegels et al, 1982). In the recent years, capture assays (Mueller-Eckhardt et al,1989) were developed, using immobilized platelet membrane glycoprotein (GP) carrying the antigens under investigation to avoid using whole platelets. These assays overcome the difficulties in differentiating platelet-reactive antibody mixtures and allow determination of antibody specificity.

It is known that previous pregnancies predispose to the production of multispecific HLA antibodies during transfusion therapy (Brand, 1991). However, there is no evidence to suggest for pre-immunization due to pregnancies as a main cause for the presence of platelet-specific alloantibodies in patients presenting with multispecific HLA antibodies (Schnaidt et al.,1996).

Multispecific HLA antibodies are a prerequisite for transfusion-induced platelet-specific alloantibodies. Since only a small percentage of haematologic-oncologic patients undergoing platelet transfusion therapy develop such multispecific HLA-antibodies Brand *et al.*, (1988), Schnaidt *et al.*, (1996) have concluded that frequency of platelet-specific alloantibodies and their impact on platelet refractoriness with regard to all patients transfused was low. They also believed that, nonimmunological factors were more often implicated in platelet refractoriness than platelet-specific alloantibodies. For

the group of highly HLA-immunized patients in need for HLA-matched single donor platelets, however, there are only as many as 25% will have additional platelet-specific alloantibodies. These platelet-specific alloantibodies will prohibit satisfactory transfusion results if HLA-matched but HPA-incompatible platelets are transfused.

5.2 Outcome of platelet transfusion in alloimmunized patients

Patients who were transfused on multiple occasions with red cells or platelets may develop platelet-reactive alloantibody and experience decreased clinical responsiveness to platelet transfusion (Sandler, 1997). Platelet transfusion refractoriness is a major complication of long-term platelet supportive care. Refractoriness lead to fatal bleeding complications in thrombocytopenic patients. Major factors involved are factors related to the clinical condition of the patient as well as HLA alloimmunisation. Patients with a history of pregnancy or non-leucocyte-depleted transfusion form HLA antibodies in high proportion (up to 50%). HPA antibodies play a minor but relatively important role in patients with HLA antibodies (Novotny, 1999).

The impact of ABO compatibility on post-transfusion platelet recovery, has been demonstrated (Lee et al., 1989). There were also similar effects of ABO compatibility on transfusion responses have been observed in patients who were refractory to random-donor platelets and received HLA-matched single-donor apharesis platelet transfusions (Duquesnoy et al., 1979).

In this study, one (1%) in seven (7.4%) alloimmunized patients died due to sepsis secondary to Acute Myeloid Leukaemia (on chemotherapy). In this patient, platelet count was noted to be on low count (< 10 x 10^9/l) within 1 hour post-transfusion although she had received > 100 units of platelet transfusions. The outcome of the other patients as shown in Table 4.1d was variable. Patient 2 showed good platelet recovery post transfusion with no evidence of bleeding tendencies and the others platelet recovery was poor with bleeding tendencies although infrequent in some of them.

5.3 Factors influencing alloimmunization

Although HLA alloimmunization is the most frequent immunological complication of platelet transfusion a significant number of patients receiving multiple platelet and red cell transfusions do not develop anti-HLA antibodies.In this study, there was no anti-HLA antibody was detected. This is because, anti-HLA was best detected using other method such as Lymphocytotoxicity test (LCT). It is therefore of interest to learn which clinical factors influence transfusion-induced platelet alloimmunization. Variables that have been identified include antigenic load, underlying disease, and chemotherapy regimen. Although antigenic load appears to be an influencing factor, the relationship between number of platelet transfusions and HLA alloimmunization remains controversial. There are probability due to the heterogeneity of patient populations in

terms of underlying disease, immunosuppressive treatment, and prior sensitization to HLAs by transfusions and/or pregnancies (Kao et al., 2000)

The effect of underlying disease on HLA alloimmunization differs in patients with different haematological disorders. Patients with aplastic anaemia have higher frequencies of HLA alloimmunization (80 to 90%) than do patients with haematological malignancies (40 to 60%). Patients with acute myelogenous leukaemia undergoing induction therapy are more likely to develop HLA alloimmunization than are patients with acute lymphoblastic leukaemia (44% vs 18%). The development of HLA antibody also occurs earlier in patients with acute myelogenous leukaemia (Pamphilon et al., 1989). These differences may be attributed to the varying degrees of immunosuppression resulting from the disease process itself or from treatment with immunosuppressive steroids which may disallow a proper immune response to be mounted. Genetic predisposition may also play a role, as illustrated by a study conducted by Taaning *et al.*, (1997) in patients undergoing open heart surgery and without significant immunosuppression was found that, 74% of patients developed cytotoxic anti-HLA antibodies after perioperative platelet and red cell transfusions.

Five (5.3%) of alloimmunized patients in this study were females and two (2.1%) were males (p=0.275). Two (2.1%) of these 5 alloimmunized patients showed non-specific patterns of alloantibodies and the other 3 patients (3.2%) were anti-HPA-5b positive. The possibility of higher percentage of female patients to be alloimmunized is probably due to prior sensitization by previous pregnancies although transfusion induced alloantibody

cannot be ruled out. Four out of five female patients (Table 4.1c) age between 39 to 50 years old although it was not considered as childbearing age sensitization by previous pregnancies can be considered.

The ages of the patients in this study ranged from 3 years to 90 years (mean of 40.3 years). Platelet alloantibody was found in 3 (3.15%) of patients less than 40 years of age and 4 (4.2%) were above 40 years of age (p= 0.807).

The Malay populations were contributed about 92.6% and for the non-Malay were around 7.4%. All the alloimmunized patients were Malay (7.4%) (p= 0.999). Statistically this was not significant. Out of 95 patients, only 7 of them were non-Malay. This is perhaps in Kelantan, majority of the populations are Malays, with few other ethnic groups.

Many plasma proteins carry polymorphic antigenic epitopes, recipients of platelet concentrates can become immunized to donor plasma proteins. The antibodies formed against plasma proteins can cause immediate-type hypersensitivity reactions with varying degrees of severity. Plasma also contains approximately 1μg/mL of HLAs, which are present in two to four different molecular forms. These different forms of HLAs are derived from the shedding of intact HLAs from cell membrane, secretion of the alternatively spliced and water-soluble form by circulating leucocytes, and proteolytic degradation of cell surface HLAs.

Although plasma HLAs have been shown to induce low titers of antibodies in some transfusion recipients, they are not very immunogenic and do not play a major role in transfusion-induced HLA alloimmunization. Because plasma HLAs in donor units can neutralise anti-HLA antibodies, these HLAs theoretically could protect the transfused platelets from anti-HLA antibodies in alloimmunized patients. However, this potential benefit in alloimmunized platelet transfusion recipients yet has to be demonstrated.

In this study, four (4.2%) of alloimmunized patients received platelet transfusions less than 20 unit and another 3 (3.2%) more than 20 units ($p= 0.927$).

However, the refractoriness to platelet transfusion cannot be ascertained because of the problem of identification of the exact time of post-transfusion platelet count measurement.

Reports about frequency and specificity of platelet-specific alloantibodies in multitransfused patients are rare. This is due to the fact that in such patients platelet-specific antibodies will occur rarely without HLA antibodies, thus complicating their identification and especially their specification (Murphy et al., 1990).

LIMITATIONS

This was a pilot study for this topic and at the samples must be 10% from the calculated sample size. Time frame for this study was short due to delay in ethical and grant approval. Our study has achieved the expected number of the sample however the company has terminated the confirmatory tests before we managed to complete our confirmatory part.

CONCLUSIONS

Alloimmunization in polytransfused patients is a well known observation which often results in decreased responses to subsequent platelet transfusions and failure to achieve haemostatic levels of platelets that may preclude these patients from important procedures, including bone marrow transplantation. Our data showed a significant rate of platelet alloimunization (7.4%) in multiply transfused thrombocytopenic patients. Perhaps more samples are needed to analyse the full spectrum of platelet alloimmunizations, especially platelet specific alloantibody.

There was also no significance relationship of platelet alloantibody and age, race and gender of patients. Number of plasma and packed red cell received by patients also showed no significance relationship.

From our data, the most frequent platelet alloantibody was anti-HPA-5b. For future transfusion, we recommend that patients receive compatible platelets after ruling out nonimmune, autoimmune and drug-related causes of platelet refractoriness since compatible platelets can significantly improve the platelet recovery.

Chapter 6

APPENDIX

BAHAGIAN PENYELIDIKAN PPSP, KAMPUS KESIHATAN USM

Tajuk Kajian: Kajian prevalen dan risiko-risiko platelet alloantibodi terhadap pesakit-pesakit yang menerima pemindahan darah di HUSM.

Nama Nama Penyelidik

Dr Rapiaah Mustafa
Dr Wan Haslindawani Wan Mahmood

BORANG MAKLUMAT DAN KEIZINAN PESAKIT (PROJEK PENYELIDIKAN)

Borang Maklumat dan Keizinan Pesakit yang digunakan dalam Projek Penyelidikan ini mengandungi maklumat berikut:-

- Tajuk Kajian/ *Topic of Research*
- Pengenalan/ *Introduction*
- Tujuan Kajian/ *Purpose of the Study*
- Kelayakan Penyertaan/ *Qualification to Participate*
- Prosedur-prosedur Kajian/ *Study procedures*
- Melaporkan Pengalaman Kesihatan/ *Reporting of Health Experiences*
- Rawatan Lain/ *Other treatments*
- Penyertaan dalam Kajian/ *Participation in the Study*
- Pampasan dan Rawatan untuk Kecederaan/ *Treatment and compensation for injury*
- Manafaat yang Mungkin/ *Possible Benefits*
- Bayaran Doktor (Penyelidik)/ *Investigator Payment*
- Soalan/ *Questions*
- Kerahsiaan/ *Confidentiality*
- Tanda Tangan/ *Signatures*
- Halaman Tandatangan/ *Signature pages*

LAMPIRAN E

Borang Maklumat dan Keizinan Pesakit

Tajuk kajian: Kajian prevalen dan risiko-risiko platelet alloantibodi terhadap pesakit-pesakit yang menerima pemindahan darah di HUSM.

Pengenalan

Anda dipelawa untuk menyertai satu kajian penyelidikan secara sukarela yang melibatkan ujian darah iaitu ujian darah platelet alloantibodi.Sebelum anda bersetuju untuk menyertai kajian penyelidikan ini, adalah penting anda membaca dan memahami borang ini. Ia menghuraikan tujuan , prosedur , manfaat, risiko, ketidakselesaan dan langkah berjaga-jaga kajian ini. Ia juga menghuraikan prosedur alternatif yang terdapat untuk anda dan hak anda untuk menarik diri dari kajian ini bila-bila masa. Sekiranya anda menyertai kajian ini, anda akan menerima satu salinan borang ini untuk disimpan sebagai rekod anda.

Tujuan Kajian

Kajian ini bertujuan untuk menentukan samada pesakit yang mempunyai bilangan platelet yang rendah (dikira dengan menggunakan mesin selepas sedikit darah diambil untuk diperiksa) dan selepas pemindahan darah akan terbentuk antibody terhadap platelet .Platelet alloantibodi adalah antibody terhadap platelet yang terbentuk selepas pemindahan darah. Antibodi ini akan terbentuk selepas menerima pemindahan darah yang mengandungi sedikit sel darah putih (samada sel darah kejap ataupun platelet itu sendiri).

Kelayakan Penyertaan

Doktor yang bertanggungjawab dalam kajian ini telah ditugaskan untuk membincangkan dengan anda/penjaga kelayakan untuk anda menyertai kajian ini. Adalah penting anda berterus-terang dengan doktor tentang sejarah kesihatan anda. Anda hanya boleh menyertai kajian ini sekiranya memenuhi syarat-syarat kelayakan berikut :

1. Anda telah mengalami thrombocytopenia (bilangan platelet yang rendah kurang dari 100,000).
2. Anda telah menerima pemindahan darah sekurang-kurangnya dua kali.
3. Menerima pemindahan darah sekurang-kurangnya dua minggu sebelum kajian dijalankan.

Prosedur-prosedur kajian

Pesakit pesakit yang menerima transfusi darah lebih dari dua kali akan diambil darah dan ujian Full Blood picture (gambaran darah yang lengkap) akan dijalankan.Ujian ini adalah untuk melihat dan mengira sel platelet yang terdapat di dalam sirkulasi darah.. Ujian kedua adalah menguji kewujudan antibody terhadap platelet (dengan menggunakan kit khas).

Risiko

Tiada sebarang risiko yang akan dialami.

Melaporkan Pengalaman Kesihatan

Jika anda mengalami apa-apa kecederaan, kesan buruk atau apa-apa pengalaman yang luar biasa, pastikan anda memberitahu jururawat atau doktor yang merawat secepat mungkin.

Rawatan Lain

Anda masih boleh menyertai kajian ini walaupun anda sedang mengikuti rawatan /kajian untuk penyakit-penyakit lain.

Penyertaan Dalam Kajian

Penyertaan anda dalam kajian ini adalah sukarela. Anda boleh menolak penyertaan dalam kajian ini atau anda boleh menamatkan penyertaan anda dalam kajian ini pada bila-bila masa , tanpa sebarang hukuman atau kehilangan sebarang manafaat yang sepatut diperolehi oleh anda.

Pampasan dan Rawatan untuk kecederaan

Jika jagaan anda mengalami sebarang kecederaan fizikal kerana prosedur yang berkaitan langsung dengan kajian ini dan anda tidak dilindungi oleh sebarang insuran, segala perbelanjaan rawatan berkaitan dengannya akan diberikan secara percuma oleh pihak Hospital.

Manafaat yang mungkin

Anda tidak perlu membayar alatan dan prosedur dalam kajian ini. Anda mungkin menerima maklumat tentang kesihatan anda daripada pemeriksaan fizikal dan ujian makmal yang bakal dilakukan dalam kajian ini.

Soalan

Sekiranya anda mempunyai sebarang soalan mengenai kajian ini atau mengenai hak-hak anda, sila hubungi doktor-doktor yang namanya tertera diatas di alamat:

Dr Wan Haslindawani Wan Mahmood
Unit Hematologi,
Hospital Universiti Sains Malaysia.
Kubang Kerian
Kelantan

Tal: 09 7664636

Atau 012-9281102

Kerahsiaan

Maklumat kesihatan anda akan dirahsiakan oleh doktor dan kakitangan kajian dan tidak akan didedahkan kepada umum melainkan dikehendaki undang-undang dan dipersetujui oleh Jawatankuasa Etika.

Data-data yang diperolehi daripada kajian ini yang tidak mengenal anda secara individu mungkin akan dibincang dalam forum atau seminar yang melibat para doktor dan mungkin akan diterbitkan dalam mana-mana jurnal perubatan. Rekod perubatan asal anda mungkin akan di semak oleh Lembaga Penyemakan Etika untuk tujuan mempastikan kesahihan prosedur dan data-data.

Dengan menandatangani borang persetujuan ini, anda membenarkan penelitian rekod, penyimpanan maklumat dan pemindahan data seperti yang diturakan diatas.

Tanda tangan

Sebagai tanda memberi kebenaran anda turut serta dalam kajian ini, anda atau wakil sah anda perlu menandatangani serta meletakkan tarikh dalam halaman tandatangan (Lihat Lampiran 1)

Borang Maklumat dan Keizinan Pesakit
Lampiran 1
Halaman Tanda Tangan

Untuk membolehkan anda menyertai kajian ini, anda atau wakil sah anda perlu menandatangani mukasurat ini.

Dengan menandatangani mukasurat ini saya mengesahkan bahawa:

Saya telah membaca semua maklumat dalam borang maklumat dan Keizinan pesakit ini, saya telah pun diberi masa yang mencukupi untuk mempertimbangkan maklumat tersebut.

Semua soalan-soalan saya telah dijawab dengan memuaskan.

Saya , secara sukarela bersetuju untuk menyertai kajian penyelidikan ini, mematuhi segala prosedur kajian dan memberi maklumat yang diperlukan kepada doktor, jururawat dan juga kakitangan yang lain yang berkaitan apabila diminta.

Saya boleh menamatkan penyertaan saya dalam kajian ini pada bila-bila masa.

Saya telahpun menerima satu salinan Borang Maklumat dan Keizinan pesakit untuk simpanan peribadi saya.

_____ _____

Nama Pesakit (ditera atau ditaip) Nombor daftar pesakit

_____ _____

Tanda tangan pesakit No Kad Pengenalan

_____

Tarikh (dd/MM/yy)

_____

Nama individu yang mengendalikan Perbincangan Keizinan (ditera atau ditaip)

_____ _____

Tanda tangan individu yang mengendalikan Tarikh (dd/MM/yy)

Perbincangan keizinan

Nama saksi dan tandatangan

Tarikh (dd/MM/yy)

Chapter 7

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POSTER PRESENTATION

1. Title: The prevalence study of Platelet Alloantibody in Multiply Transfused Thrombocytopenic Patients in HUSM.

   Venue: 5th Malaysian National Haematology Scientific Meeting. Putrajaya Marriott, Kuala Lumpur

   Date: 9th –11th April 2004.

2. Title: The prevalence study of Platelet Alloantibody in Multiply Transfused Thrombocytopenic Patients in HUSM.

   Venue: 9th National Conference on Medical Sciences. School of Medical Sciences, Universiti Sains Malaysia.

   Date: 22nd – 23rd May 2004.

3. Title: The prevalence study of Platelet Alloantibody in Multiply Transfused Thrombocytopenic Patients in HUSM.

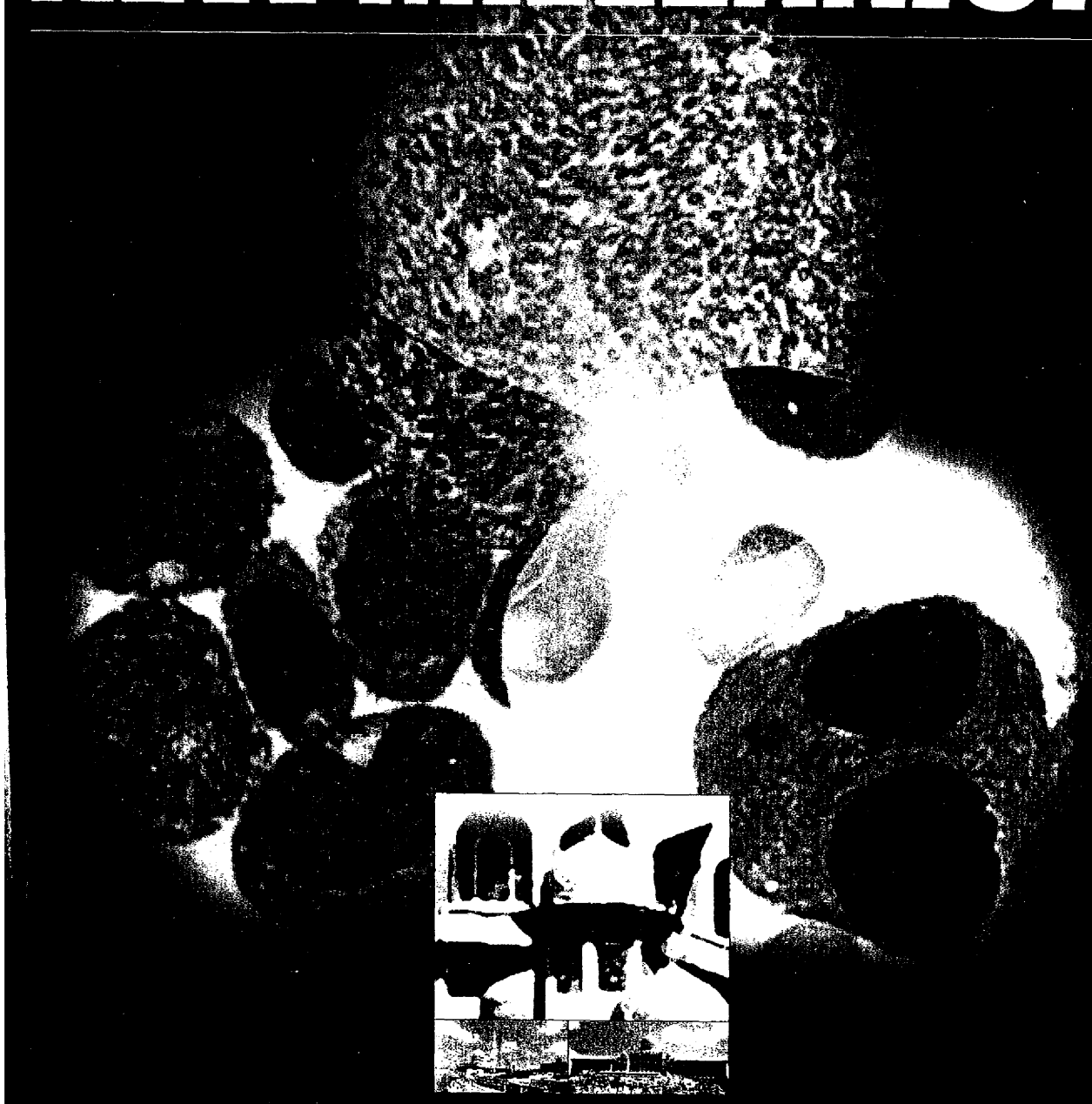
   Venue: Experimental Biology 2005 and the XXXV International Congress of Physiological Sciences, San Diego, California.

   Date: 31th March - 5thApril 2005.

The Malaysian Society of Haematology
Persatuan Hematologi Malaysia

5th Malaysian National Haematology Scientific Meeting

HAEMATOLOGY IN THE NEW MILLENNIUM



9th - 11th April 2004
Putrajaya Marriott

Study Of Prevalence And Risk Factors Of Platelet Alloantibodies In Multiply Transfused Thrombocytopenic Patients In HUSM

TT01

Department of Haematology, Hospital University Sains Malaysia
M Rapiaah
W M W Haslindawani

Background

Multiply transfused patients are frequently subject to alloimmunization which was differently found in the literature .These platelet alloantibodies can result in refractoriness to platelet transfusion . Aim of the study was to detect the presence of platelet alloantibodies in multiply transfused thrombocytopenic patients in HUSM.

Methods

Sixty two thrombocytopenic and multiply transfused patients were recruited prospectively . The blood were subjected to test using Solid Phase system (Capture P).

Results

There were 32 males and 30 females (1:1) .The age of the patients were between 3-85 years old. The frequency of transfusion were between 2-15 . Four of the patient (6.4%) were detected to have platelet alloantibodies.

Discussion

These findings have important implications for the selection of platelet donors for alloimmunized recipients.

9th NATIONAL CONFERENCE ON MEDICAL SCIENCES

LIGHTS OF MEDICINE

USM
UNIVERSITI SAINS MALAYSIA

22 - 23 MAY 2004

SCHOOL OF MEDICAL SCIENCES , UNIVERSITI SAINS MALAYSIA

STUDY OF PREVALENCE AND RISK FACTORS OF PLATELET ALLOANTIBODIES IN MULTIPLY TRANSFUSED THROMBOCYTOPENIC PATIENTS IN HUSM

Authors : *W Haslindawani W M , Rapiaah M, **Shafini M.Y.***

Institution : *Department of Haematology , Hospital University Sains Malaysia*

Background :

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Disscussion :

These findings have important implications for the selection of platelet donors for alloimmunized recipients