PLATELET ALLOANTIBODY SPECIFICITIES IN MULTIPLY TRANSFUSED PATIENTS

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CERTIFICATE

This is to certify that this dissertation entitled "**PLATELET ALLOANTIBODY SPECIFICITIES IN MULTIPLY TRANSFUSED PATIENTS"** is the bonafide original work done by **Dr. B.K.. MADHAN KUMAR,** Post graduate in Immunohaematology & Blood Transfusion, under my overall supervision and guidance in the department of Transfusion Medicine, The Tamil Nadu Dr. M.G.R. Medical University in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Immunohaematology & Blood Transfusion,** during the period of June, 2005 to March, 2008**.**

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KEY TO ABBREVIATION

HLA	-	Human leukocyte antigen
HPA	-	Human platelet antigen
ELISA	-	Enzyme linked immunoassay
GP	-	Glycoprotein
AML	-	Acute myeloid leukemia
ALL	-	Acute lympoblastic leukemia
ADP	-	Adenosine Diphosphate
ATP	-	Adenosine tri phosphate
PIET	-	Platelet immunofluoresence test
PNPP	-	p- nitrophenoyl phosphate
MDS	-	Myelodysplastic syndrome
VWF	-	Von Willebrand disease
RDP	-	Random donor platelet
SDP	-	Single donor platelet

CONTENTS

INTRODUCTION

Transfusion plays an important role in the management of patients with hematological malignancies and disorders. Patients during the induction chemotherapy may need multiple transfusion of platelets and red cells and may develop refractoriness; platelet count increment deteriorate even after adequate dose of platelets are transfused. Although several studies reported non-immunological factors like fever, Splenomegaly, septicemia and severe bleeding are responsible for refractoriness in some patients, no specific reasons can be found. This raises the suspicion of platelet antibodies for the cause of refractoriness. Till now in many studies, the presence of HLA and platelet specific antibodies were found to be between 10%-60%in the patients who were treated with multiple transfusions.

Non- immunological factors in impairment of post transfusion platelet count increment are difficult to influence, but refractoriness due to platelet alloimmunization can be prevented and treated by using HLA and HPA matched transfusion, platelet cross matching by ELISA, leukocytes filtered and irradiated transfusions. This possibility paved way for the development of immunological techniques to detect platelet alloantibodies against HLA class 1 antigens, platelet glycoproteins and human platelet antigens.

REVIEW OF LITERATURE

Bajpai M, et al tested for antibodies in 50 patients before transfusion and 3-4 weeks after multiple transfusion, found 66% with antiplatelet antibodies of which 71% were not responding to transfusion. 26% of antibodies negative patients were non responsive. (National medical journal of India 2005 may 18 issues 3, 134- 6)

Los Sc, et al did a study on 60-thalassemia patients and found 19 with (31%) HLA, 13with (22%) with both HLA and HPA antibodies and 1with (2%) HPA antibodies. The follow up study showed that 7 patients developed HLA antibodies and 1 lost the antibodies. 9 patients developed HPA antibodies and 12 lost at least one HPA antibodies. He concluded RBC transfusion can induce alloimmunization and HLA antibodies are sustain longer than HPA antibodies. (Transfusion 2005 may 45, I 5, 761-5)

Kiefel v et al tested 252 patients with hematological malignancies, Hematological disorders with thrombocytopenia, MDS, chronic Myeloproliferative disorders and found 108 with (42%) HLA antibodies and 20 with (8%) HPA antibodies positive. He observed 53% of female patients with previous pregnancy and 23% without previous pregnancy were positive for antibodies. (Transfusion 2001 June 41(6) 776- 70)

According to **Cristina sanz** studies 3.8% of the HLA alloimmunization patients are positive for HPA antibodies glycoprotein iib/iiia and anti HPA-5b and concluded HPA antibodies in HLA immunized patients is very low and can be searched only when other causes are ruled out. (Transfusion 2001 volume 41(6) 762-5)

Lo sc et al studied on 103 patients and found 40 are platelet reactive antibodies positive of which 22 had both HLA and HPA antibodies, 12 had HLA alone and 5 had only HPA antibodies. 81% of platelet specific antibodies were found in HLA alloimmunized patients of which frequently found were glycoprotein ia/iia and iib/iiia. (J formos med assoc 2000 dec; 99 (12): 902-5)

Pereira j et al observed alloimmunization in 17% and 2% due to HLA and HPA in adult patients and no children were reported positive for antibodies after every four weeks or whenever the 1 hr corrected count increment for platelet transfusion was lower than 5000. (Rev med chill 1997 Nov; 125(11): 1305-12)

40 patients had at least one episode of refractoriness out of 145 patients who received more than three single donor platelet transfusions, in whom 15 refractory patients show alloantibodies . 7 patients got inadequate transfusion response due to Alloimmunization alone and 8 due to alloimmunization and fever Or sepsis (**Legier TJ et al.** Ann hematol 1997 apr; 74; 4; 185-9)

Taaning E et al studied 117 heart surgery patients for HLA antibodies and found 21 were positive, 18 were negative before transfusion and 2 cases positive for platelet specific

antibodies who received multiple transfusion .(vox sang 1992; 72; 4; 238-41)

Pan reactive antibodies against Gpib/ix has similar frequency as that of Gpiib/iiia and Gpia/iia always in combination with above once. Most HPA antibodies were directed against HPA-Ib and –5b in **Kurz m et al** (Br j of hematology 1996; dec; 95; 3; 564-9)

Yan et al screened antibodies for HLA class I and GP iib/iiia by using GTI MACE 1, in which 1.25% Glycoprotein antibodies and 75% for HLA antibodies are positive. (Journal of thrombosis & hemostasis 2005 vol 3,sup 1)

Uhrynowska M et al screened 104 multi-transfused patients were 31% are alloantibodies positive of which HLA alloimmunization is 20%, HPA - 1a, 2b, 3a and 5b in less than 4% and 6% of GP ii/iiia. Eur jor of hematol 1996 apr 56; 4; 248-51.

Hogge DE et al concluded that alloimmunization and refractoriness is 37% in pediatric patients and low in acute myelogeneous Leukemia. The detection of antibodies predicts poor response to random donor platelet transfusion, but most such patients improved with HLA matched platelets. (Transfusion 1995; aug; 35; 8; 645-52)

Novothy VM et al published HLA alloimmunization in high risk negative patients is 2.7%(3 of 112) and 31%16 of 54) in positive risk patients. 22% positive for alloimmunization when stored platelet transfused by bed side filtrations. (Blood vol 85; 7 april 1995; 1736-41)

Chow MP et al found disappearance of platelet antibodies during follow up period by 50% of positive cases of HPA and 10% in HLA antibodies cases. Acta hematol 1994; 92; 2; 57-60.

In 59 multi transfused patients treated for hematological disorders, 10(17%) were found to be positive for human platelet specific antibodies. 6 patients with HPA-1b, 3 with HPA-3a, 2 with HPA-1b and 3a and 1 with HPA-5a. (**Schnaidt m et al** Beitr infusionsther transfusion medicine 1994; 32; 240-3)

Ishida A et al studied platelet transfusion efficacy after hematopoietic transplantation in 42 patients and found platelet alloimmunization due to HLA and HPA antibodies is 17% and 14% in allogeneic and autologous transplantation patients with platelet transfusion. (Transfusion, 1998;sep; 38; 9; 839-47)

HLA antibodies disappear in 6 patients who discontinued transfusion and 8 patients after treating with matched and leukocyte reduced platelet transfusion out of 40 patients alloimmunized.(**Murpy MF et al** Br J of hematol 1987;nov; 67; 3; 255-60)

Kurz M et al reported MAIPA assay is sensitive method than LCT in detecting platelet reactive HLA antibodies (24%vs 8%). (Br j of Hematology Volume 95(3-I), December 1996, pp 564-569)

Frequency of alloimmunization in acute non-lymphocytic leukemia is lower than aplastic anemia and no relationship between Alloimmunization development and number of platelet transfused according to **Nozaki H et al.** (Tokai J exp clin med1986; aug; 11; 3; 201-4)

Mc Grath K et al study states that platelet recovery at 1hr and 20hr post transfusion was not significantly reduced in the presence of platelet specific antibodies. (Br j of hematology 1988;mar; 63 3; 345-50)

HLA homozygous allies have high prevalence of platelet alloimmunization and antibodies to certain specificities like HLA B60 and 75 are in higher rate in **Chop Mp et al.** (Zhongua yi xue zazhi 1993 may; 51; 5; 329-32)

85% of multiple RBC transfused patients are alloimmunization to HLA or HPA Antigens in **Friedson DF et al.** (Blood 1996;Oct; 88; 8; 3216-22)

Chop MP et al, development of platelet alloimmunization from initial transfusion may vary from 10-190days and 40% who did not develop antibody will never develop later. (Zhongua yi xue za zhi 1991;apr;47;;4;237-41)

Mac Pherson BR concluded that 79% antibodies found inMultiple transfusion patients are directly against public HLA Antigens. (Ann clin lab sci 1986 Jan; 16; 1; 38-44)

Wernet D et al studied 388 patients for HLA and HPA antibodies of which 27 showed cold reacting auto antibodies and lymphocytic restricted reaction in 20 patients. (Vox sang 1993; 65; 2; 108-13)

28% (8 out of 29 patients) of patients developed HLA antibodies after ventricular Assist device support and need of more transfusion in post Operative by **Mc kenna DH et al**. (J heart lung transplant 2002;nov; 21; 11; 1218-24)

Presence of platelet antibodies was significantly associated with post transfusion fever in **Masanoir S et al.** (Japanese J of clinical oncology 1977; 7; 35-43)

Splenectomized patients have greater platelet increment than normal sized spleen patients at 1hr and 24hr after transfusion in **Sherrill et al**. (Blood may 2005; 105; 10; 4106-15)

Docoteau et al. Only 2.3%of ALL children's presented with platelet antibodies than 31% of AML. (J of pediatric hematology oncology 1995; nov; 17; 4; 306-10)

Godeau B et al studied 50 patients who received multiple platelet transfusion, only 2 patients had HPA antibodies of which 1 showed poor platelet recovery and 11 were positive for Para -formaldehyde dependent platelet with out refractoriness.(Br J of Haematol 1992;jul; 81; 3; 395-400)

Lam CP published A2, A11, B16 and B60 are specificities of the identified antibodies in alloimmunization patients.(Zhon yi xue za zhi 1992; Dec; 5; 6; 329-42)

PLATELETS

PLATELETS STRUCTURE & FUNCTION: -

Platelets are small, anucleated fragments measuring 2micro m in diameter and with volume of 8-10 FL. The platelets vary in number, size, density, age and apparent physiologic effectiveness. The life span of the platelet is around 8-12 days and turnover is estimated to be 1.2 to 1.5x 10*11 cells per day. (**Wintrobes clinical hematology** 11th edition.)

It contains membranes, microtubules and granules. The external membrane composed of proteins, glycoproteins and mucopolysaccharides, which play major role in mediating platelet response to stimuli and in expressing antigenic characteristics. The internal membrane contains sub membranous microfilaments, which maintain the platelet discoid shape.

The microtubules are an enclosed system containing ionized calcium, enzymes and actin, myosin filaments. These are involved in motor activities such as pseudopodia movements and secretion.

The granules in organelle zone are i. Delta granules like ADP, ATP and serotin etc ii. Alpha granules like platelet specific proteins, platelet factor 4, fibrinogen, factor v etc. these granules are found dispersed in cytoplasm of platelet, and on stimulation granules are released into extra cellular environment. (**Anderson and ness**, scientific basis of transfusion medicine.)

PLATELET ANTIGENS: -

Platelets contain variety of antigens on the cell surface,

1) ANTIGENS FOUND ONLY ON PLATELETS

Platelets Specific antigens arise as a result of polymorphisms of platelet membrane glycoprotein. These membranes contain number of glycoproteins that contribute to the immunogenic make up of the platelet. (This HPA nomenclature system was adopted in 1990 **Vodem Borne AE, Decary F** 1990).

The intergrins are membrane glycoprotein heterodimers, consisting of non -covalently associate alpha & beta subunits. There are 17 alpha & 8 beta subunits, with the possible alpha beta combination led to discover 23 different intergrins in humans. Two platelet membrane receptors that are seen prominently in antigenic profile of platelets, the cohesion receptor αiibβ3 and the collagen receptor α2β1 are intergrins. The platelet intergrins αiibβ3 are initially called as glycoprotein iib/iiia which helps in platelet aggregation (The common cohesive pathway) by binding of adhesive proteins fibrinogens & von wille brand factor (vwf). The platelet collagen receptor α2β1, intergrins is initially called as Gp ia/iia, which plays a fundamental role in adhesion of blood platelets to both fibrillar (types i, ii, iii and v) and non fibrillar (type iv, vi, vii, viii) collagens. Third receptor complex Gp ib-ix-v is a heptamer contains 1 molecule of glycoprotein v and 2 molecules of each glycoprotein Ib, IIb and ix. This complex is a receptor for vwf. Platelet glycolipids plays important role in structural and functional characteristics of platelet membrane but less know. Lactosyl ceramide represents major glycolipids in platelet membrane.

The HPA Nomenclature categorizes all antigens expressed on the platelet membrane, except those encoded on genes of major histocompatability complex. HPAs are grouped in systems based on having all antibodies defining a given alloantigen and its antithetical alloantigen. To date , 24 platelet specific alloantigen (tab 1) have been defined of which 12 are grouped into six biallelic systems and for the remaining 12 alloantibodies are yet to be discovered (**Metcalfe P el al** 2003).

Table- 1

Human platelet antigens

System	Antigen	Glycoprotein	CD
HPA-1	HPA-1a	GPIIIa	61
	HPA-1b	GP	
HPA-2	HPA-2a	GPIb	42b
	HPA-2b	GP	
HPA-3	HPA-3a	GPIIb	41
	HPA-3b	GP	
HPA-4	HPA-4a	GPIIIa	61
	HPA-4b	GP	
HPA-5	HPA-5a	GPIa	49b
	HPA-5b	GP	
	HPA-6bw	GPIIIa	61
	HPA-7bw	GPIIIa	61
	HPA-8bw	GPIIIa	61
	HPA-9bw	GPIIb	41
	HPA-10bw	GPIIIa	61
	HPA-11bw	GPIIIa	61
	HPA-12bw	GPIa	42c
	HPA-13bw	GPIa	49b
	HPA-14bw	GPIIIa	61
HPA-15	HPA-15a	CD109	109
	HPA-15b		
	HPA-16bw	GPIIIa	61

Platelet antigens are also present in other cells, HPA IA has been detected on vascular smooth cells & fibroblasts, and HPA-5 also expresses on activated T cells.

2) **HLA ANTIGENS ON THE PLATELET SURFACE**

The principal antigens shared by platelets and lymphocytes are HLA class 1 and 2 Antigens, where platelet are the major source of class I HLA antigens. The studies suggested that these antigens were not only firmly embedded in the membrane and but also absorbed from the surrounding plasma (**kao KJ et al 1988 and sugawara S et al 1987**). Recent studies showed that most class 1 HLA molecules on platelets are integral membrane proteins, which are persisting from the megakaryocyte stage of development. Platelet process m RNA encoding HLA class 1 molecules, which are capable of synthesizing HLA molecules (**Mollision** 11^{TH} Edition). Peptides of the HLA molecules derived from the megakaryocyte platelet specific GP ix. The number of HLA molecules expressed on the platelet surface various from 15,000-1,20000. (HLA class II antigens are not detectable on platelets but HLA -DR antigens can be induced on platelets surface by stimulation with cytokines Harvey G. Klein & David J. Anstee. **Mollision** 11^{th} edition)

3) RED CELL ANTIGENS ON PLATELET

Red cell antigens ABH, Lewis, I, & P are also expressed on platelets. These antigens are intrinsic and others are absorbed from plasma. This antigen could explain reduction in platelet recovery when ABO incompatible platelets are transfused. The ABH and other red cell antigens are expressed on many integral platelet membrane Glycoproteins including GP II b, GP IIIa,

GD IV, V, GP Ib /IX & GP ia\ IIa.(**Curtis BR et al 2000, Santoso S et al 1991**)

PLATELET ANTIGEN ALLOIMMUNIZATION: -

Alloimmunization is an immunological state referring to immune system's response to foreign antigens most often involving the production of antibodies directed at the antigens. The platelet alloimmunization leads to clinical condition PLATELET REFRACTORINESS in which a patient does not achieve the anticipated platelet count increment from a platelet transfusion. An immune or a non-immune condition may cause refractoriness.

I) IMMUNOLOGIC BASIS OF PLATELET REFRACTORINESS

1) ALLOIMMUNIZATION TO HLA ANTIGENS

Recent practice of prophylactic multiple platelet transfusion, results in decreasing effectiveness in many patients due to induction of alloantibodies to HLA class I, HPA and other Red cell antigens. HLA Antibodies are not naturally occurring Antibodies and develop due to pregnancy and Blood Transfusion. Development of HLA antibodies occur as early as 10 days after primary exposure or 4 days after secondary exposure in patients who had been multiple transfused or multiple pregnancy(**Rossi's** principles of transfusion medicine 3rd edition).The HLA alloimmunization is related to underlying diseases, immunosuppressive effect and immune responsiveness. There is some general agreement that primary alloimmunization to HLA Antigens is unlikely to occur before 3-4 weeks after the first transfusion in patients receiving multiple transfusions, and antibodies detected sooner may be due to secondary immune response. The major immunizing source of HLA antigens in transfused platelets and red cells is the donor leukocytes. In some patients presence of HLA class I Antibodies does not produce refractoriness to platelet transfusion due to low frequency HLA antigens and

antibodies against them will not react with the platelet of random donors. Some donor antigens express so weakly that makes platelet to survive normally even though patients have antibodies. Further more patients with HLA class I antibodies may form antiidiotype antibodies, which will inactivate the class I antibodies. In significant percentage of alloimmunized patients, antibodies disappear or decline with time. (**Mollisons** 11th edition)

2) ALLOIMMUNIZATION TO PLATELET –SPECIFIC ANTIGENS

HPA Antibodies have been discovered in Neonatal alloimmune thrombocytopenia, post Transfusion Purpura and multiple transfusions and are rare cause of transfusion refractoriness. Alloimmunization to high- frequency platelet specific antigens leads to a major challenge in finding compatible platelets. Patients may also from antibodies against entire platelet glycoproteins molecule V, Ib, IIb-IIIa etc.

3) ALLOIMMUNIZATION TO BLOOD GROUP ANTIGENS

Platelets have weak expression of antigen A& B. Donor with strong A or B may develop refractoriness after transfusion of ABO incompatible platelets. This is mainly due to the anti-A or B substance in patients react with donor ABH substance.

II) NON-IMMUNE PLATELETS REFRACTORINESS: -

Alloimmunization is not only the major cause of refractoriness, but non immune factors like Splenomegaly, fever, infection, DIC, hepatic dysfunction and veno-occlusive disease and marrow transplantation also play a role. Platelet refractoriness has been reported with number of medication Antibiotics, Amphotercin, GM-CSF and other interferon's.

INVESTIGATIONS FOR PLATELET ANTIBODIES:

Tests are based on the detection of platelet bound Ig. The following are the tests for the detection of platelet antibodies,

1) Platelet immunofluoresence test, (**Von dem borne** 1978)
2) The enzyme-linked immunosorbent assay (**Tata and co workers** 1977).
3) Mixed red cell adherence assay and
4) Monoclonal antibody specific immobilization of platelet antigens.

THROMBOCYTOPENIA

It is defined as a subnormal number of platelets in the circulatory blood. It occurs due to 3 processes;

A. Disorders of platelet production (decrease production or dysfunction).
B. Platelet sequestration.
C. Platelet destruction.

Decrease production occurs due to toxic (chemotherapy agents, ethanol, radiation and ganciclovir etc.) or pathological in suits damage megakaryocytes in the bone marrow like primary and secondary leukemias, myelodysplastic syndrome, met static carcinomas.

Dysfunctional platelets are normal in number, but their function is abnormal. It is caused by congenital disease like glanzmann thrombasthenia, Bernard soulier syndrome, platelet type von wille brand disease and gray platelet syndrome etc and acquired causes due to medication, uremia, cardiopulmonary bypass and hematological disorders and malignancy.

Hypersplenism due to any causes like portal venous hypertension, liver cirrhosis, infection, malignances lead to pooling of platelets in the spleen and increase sequestration. Platelet destruction occurs mainly due to immune mediated include drug induced, platelet auto or allo antibodies, ITP and non immune causes like DIC, TTP, microbial infection, trauma, invasive surgery, burns and obstetric conditions. (**Rossi's platelet transfusion**)

Thrombocytopenia is classified into three types depending on the count as (**Lawrence D Petz**, clinical practice of transfusion medicine 2nd Edition)

1. Severe – 5,000-20,000/uml.
2. Moderate – 20,000-50,000/uml.
3. Mild – 50,000-1,00,000/uml.

PLATELET TRANSFUSION: -

Early in 20th century, fresh whole blood was the only source of viable platelets, which was replaced in 1960-1970 by concentrates separated from whole blood after development 0f plastic bags. It is mainly used to treat or prevent bleeding in the patients with low platelet count.

Since platelet express ABO antigens, ABO compatible transfusion appears to give better results than incompatible once (**Duquesnoy RJ et al 1979, Heal JM et al 1987**). Small amount of red cells are contaminated in both random donor platelet (less than .5ml of RBC) and single donor platelets (less than 5ml of RBC). When Rh-positive donors are transfused into Rh-negative patients, there is 14% incidence of Rh alloimmunization, which can be prevented by

giving Rh immuno globulin intravenously.

The decision to transfuse platelets falls into two categories I)Treatment for bleeding due to thrombocytopenia irrespective of platelet count, and II)Prophylaxis for patients having count less than 20,0000/µml (**christopher D Hillyer et al** Hand book of pediatric transfusion medicine).

The dose of platelet for transfusion is determined by number of platelet in single donor platelet ($3x10^{11}$/unit) and random donor platelet ($5.5x10^{10}$/unit). Each unit of RDP increases 5000-10000/µml in a 70kg adult and 50,000-60,000 in SDP (**Denise M Harmening** 4^{TH} Edition). Dosing is done on the post transfusion platelet increment, the higher the post transfusion platelet increment, longer the interval between the transfusions.

MANAGEMENT OF ALLOIMMUNIZED PATIENTS: -

The refractory status, which fails to random donor platelet transfusion, ranges from 13% to 100% (**Pamphilon DH et al** 1989) of patients receiving multiple transfusions. Several methods were considered to treat such patients with 1) fresh blood, single donor, ABO compatible platelet transfusion. 2) HLA matched platelet transfusion according to cross reactive associations grade. 3) Platelet cross matching by ELISA, PIFT. 4) Treating alloimmunity status by IV immunoglobulin and 5) other factors like using vinblastine loaded platelet transfusion, treating with cyclosporin A, Immunoadsorption, plasmapheresis which all have limited successes.

Classification of donor/recipient pairs on the basis of HLA match

CROSS REACTIVE ASSOCIATIONS

A	All 4 antigens in donor identical to those in recipient
BIU	Only 3 Ag detected in donor; all present and identical to recipient
BIX	3 donor Ag identical to recipient; 4^{th} Ag cross reactive with recipient
B2U	Only 2 Ag detected in donor; both present and identical in recipient
B2UX	Only 3 Ag detected in donor; 2 identical in recipient, third cross reactive
B2X	2 donor Ag identical to recipient; 3rd and 4^{th} cross reactive with recipient
C	1 Ag of donor not present in recipient and non cross reactive with recipient
D	2 Ag of donor not present in recipient and non cross reactive with recipient

PREVENTION OF ALLOIMMUNITY: -

There is evidence that the leukocytes in the platelet concentrate are responsible for the formation of HLA antibodies than platelets, which can be prevented by removing or inactivating class 2 antigens cells. To limit the antigenic exposure, single donor platelet transfusion with leukocytes filtered can be used. By using ultraviolet B irradiation blood products can be used, which prevents the interaction of dendritic cells contained in the transfusion products with recipient T lymphocytes and reduces the formation HLA antibodies.

AIM OF THE STUDY

- ✓ To evaluate the incidence of platelet alloimmunization in multiple transfused patients.

- ✓ To compare frequency of HLA class 1 antibodies and Epitopes of Glycoprotein iib/iiia antibodies in multiple transfused patients.

MATERIALS AND METHODS

The study was conducted in the Department of transfusion, the Tamil Nadu Dr M.G.R medical university, guindy, chennai.

DESIGN OF THE STUDY: A prospective study.

PLACE OF COLLECTION:

Blood samples were collected from Cancer Institute Adyar and Government general Hospital, chennai for a period of 1 year. Detailed history and consent were taken from the patients, diagnosis and laboratory detail were taken from the case record.

INCLUSION CRITERIA:

A total of 40 patients who were treated for leukemias, aplastic anemia and lymphomas with chemotherapy and multiple transfusions and suspected platelet refractoriness were included in this study.

EXCLUSION CRITERIA:

1. Splenectomized patients.
2. Multiple pregnancies.
3. Received multiple transfusions before.
4. History of drug intake.
5. Positive for HLA class I and Gp iib/iiia epitopes antibodies.

TIME OF COLLECTION:

The first Blood samples were collected before starting first transfusion and Chemotherapy from these patients. Patients were followed during therapy who was supported with multiple Transfusion i.e. more than 10 units of platelets, packed red cell, whole blood and plasma for a period of more than 3weeks. The second blood samples were collected when post transfusion platelet count increment is less than 4500 per unit of random donor platelet after 24hr.

INVESTIGATIONS:

1) **PLATELET COUNT**:

Platelet count is done by automatic analyzer before and 24hr after transfusion.

2) **SEROLOGICAL TESTS**:

COMMERCIALLY available ELISA kit (GTI-MACE 1) was used to detect antibodies by following manufactures instructions.

MACE®I is a qualitative solid phase enzyme linked immunosorbent assay (ELISA) designed to detect IgG antibodies to HLA class I antigens and to epitopes on the platelet glycoprotein IIb/IIIa.

PRINCIPLE :

Patient serum or plasma is incubated with intact platelets allowing for antibody, if present, to bind to the platelet glycoprotein. Unbound antibodies are washed from the platelets. The antibody-sensitized platelets are solubilized by the addition of a lysis buffer containing a non-ionic detergent. The platelet lysate containing soluble glycoprotein is transferred to microwells. This allows the platelet and HLA class I Glycoprotein (sensitized or unsensitized with patient Antibody) to be captured by immobilized monoclonal antibodies. Control Samples

are handled similarly. After a brief incubation period, unbound Glycoproteins are washed away. An alkaline phosphate labeled anti-human Globulin reagent, Anti-IgG is added to the wells and incubated. The Unbound Anti-IgG is washed away and the substrate PNPP (p nitro phenyl Phosphate) is added. After a 30-minute incubation period, the reaction is stopped by a sodium hydroxide solution. The optical density of the color that develops is measured in a spectrophotometer. A positive result indicates the presence of glycoprotein-specific antibody on the captured GPIIb/IIIa or HLA glycoproteins.

SPECIEMENT COLLECTION:

SERUM: Blood sample is collected by using aseptic technique and separated serum is stored at -20 till test is performed (Samples frozen at -20°C or below remain in good condition for several years (2-3 years)). However, in order to avoid the deleterious effect of repeated freeze/thaw cycles, samples are aliquot in small volumes and then stored frozen.

NOTE:

Serum or plasma should be separated from red cells when stored or shipped only whole human serum or plasma is suitable for this assay. Microbial contaminated, hemolyzed, lipemic or heat inactivated samples may give inconsistent test results and should be avoided

METHOD:

Materials provided in the kit:

- 12-1 x 8 Microwell Strips with holder
- 1x 50 mL Concentrated Wash
- 1x 14 mL Specimen Diluents

- 1x 14 mL substrate buffer
- 1x 14 mL Stopping Solution
- 1x 80: L Anti-Human IgG Conjugate
- 6 x 50 mg PNPP Substrate
- 1x 0.7 mL Positive Serum Control
- 1x 0.7 mL Negative Serum Control
- 3 Normal Platelet Controls (50 µL Rehydrate)
- 1x 2.5 mL Cell Lysis Buffer
- 1x 50 mL Cell Resuspension and Preservative Solution
- 12 Plate Sealers
- 2 Recording Sheets

MATERIALS REQUIRED:

1. Test tubes for patient sample, and control dilutions and for reagent dilutions.
2. Transfer pipettes.
3. Adjustable micropipettes to deliver 10 - 100 µl and 100-1,000 µl and disposable tips.
4. Timer.
5. Microplate reader capable of measuring OD at 405 or 410 and 490 nm.
6. Absorbent paper towels.
7. Deionized or distilled water.
8. Microplate washer or device.
9. Centrifuge capable of separating serum or plasma from platelets.

10. 37°C water bath or incubator.

11. Micro centrifuge for pelleting platelets.

TEST PROCEDURE:

- All reagents were brought to room temperature.
- Working Wash solution was prepared by diluting Concentrated Wash, 1 volume of Concentrated Wash to 9 volumes of deionized or distilled water was added and Mixed well.
- The number of patient samples to be tested was determined.
- Using the Recording Sheet, each sample was assigned to a location consisting of one well on each strip and the identity of each sample was recorded on the Recording Sheet.

SAMPLES AND CONTROLS PREPARATION:

PREPARATION OF POSITIVE AND NEGATIVE CONTROLS:

One vial of the dried Normal Platelet Control was Rehydrate by adding 400 µmL of Cell Resuspension and Preservative Solution and Allow to stand for 10 to 30 minutes at room temperature.

Rehydrate platelets was mixed well and Centrifuge tube to pellet cells.

Supernatant was decant and blot the tubes dry. 50 µL of Cell Resuspension and Preservative Solution is added to the platelet button and mixed well by pipette to homogenous suspension.

15 µL of the normal platelet suspension was transferred into two clean micro centrifuge tubes, and 150u L of Positive Serum Control and negative serum control to each one of the tubes and mixed well. Labeled them as positive control and negative control.

PLATELET PREPARATION:

For each platelet sample to be tested, 2 – 3 mL of platelet rich plasma is centrifuged to platelet button. Supernatant plasma was discarded. To the platelet button 400 uL of Cell Resuspension and Preservative Solution was added, mixed well and Centrifuge to obtain a platelet button. Supernatant fluid is discarded. Above steps was repeated for 3 to 4 washes and after last wash supernatant was removed, tubes drained with an absorbent paper towel.

Equal volume of Cell Resuspension and Preservative Solution to the platelet button to make 50% homogenous suspension and 15 µL of the 50% platelet suspension was transferred to a clean-labeled tube.

PLATELET LYSING:

150 uL of patient serum to be tested is added to the tube containing a platelet test suspension prepared in the previous step and mix well with pipet. Samples and the controls are incubated at 37ºC for 40 minutes in dry incubator.

Test and control samples are washed twice by adding 400 uL of Cell Resuspension and Preservative Solution to each tube and centrifuging to pellet the platelets. Supernatant fluid is discarded after each wash. Blot tubes are blot with absorbent paper to remove all residual fluids, with out disturbing the platelet button after final wash.

Note:

Cell Lysis Buffer is diluted by adding 100 µL of Cell Lysis Buffer to 900 µL of

deionized or distilled water for each five platelets to be tested. Finally 180 uL of diluted Cell Lysis Buffer is added to each test and control tube and mix well with a pipet and vortex for complete Lysis. Platelet lysates are tested

ELISA FOR DETECTION:

Microwells frame are removed from pouch and strips taken depending upon the test numbers and reseal unneeded strips in the protective pouch. Since only one frame is provided in the kit frame is used for all tests. The strips, were placed in the frame and marked at the top according to Recording Sheet.

50 µL of sensitized platelet lysate, negative control and positive control lysate are added to the wells designated on the Recording Sheet leaving blank wells. The micro wells were sealed with a plate sealer and incubate for 40 minutes in a 37°C dry incubator.

Note:

Conjugate is diluted 1 to 100 in Specimen Diluents in a polypropylene container.

After incubation the contents are Aspirate and blot on absorbent toweling. To this 300-µL Working Was h solution is added in each well and washed. Above washing steps are repeated for 3 or 4 times. After final wash remove all residual wash solution by Inverting on absorbent toweling to prevent drying.

50 µL of diluted Conjugate was added to all wells except blanks, Seal the microwells with a plate sealer and incubate for 40 minutes in a 37°C dry incubator.

Note:

a. PNPP is dissolved in Substrate by adding in 0.5 mL deionized or distilled water to the vial and mix well. Protect from light until use.

b. PNPP is diluted 1 to 100 in the Substrate Buffer.

After incubation the contents are Aspirate and blot on absorbent toweling. To this 300-µL Working Was h solution is added in each well and washed. Above washing steps are repeated for 3 or 4 times. After final wash remove all residual wash solution by Inverting on absorbent toweling to prevent drying and microwells kept in dark for 30 minutes at RT (22º-25ºC)

After incubation 100 µL of the diluted PNPP solution to all the wells except blanks. Reaction is stopped by adding 100 µL of Stopping Solution to each well in the same sequence as the addition of substrate and Add 200 µL of Stopping Solution to the blank wells.

The absorbance (OD) of each well read at 405 or 410 nm using a reference filter of 490 nm by using automatic ELISA reader and the results were recorded on the Recording Sheet.

RESULT

Of the 40 patients included, 25 were males and 15 were females. The age of the patients ranged from 3 to 58yr with a mean of 25.80 and Std Deviation of 19.66. Acute leukemias (30) were the main underlying disorders. The others were chronic leukemias (4), aplastic anaemia (2) and lymphoma (4). The number of blood and/or blood components given to these 40 patients ranged from 10- 36 units for the period of more than 3-4 wks.

TABLE 1 **SEX DISTRUBUTION**

Sex	no of cases	Percent
Male	25	62.5
Female	15	37.5

FIGURE 1 **SEX DISTRUBUTION**

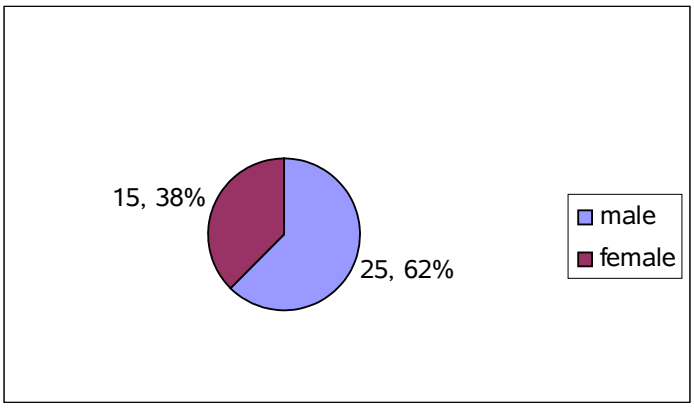


TABLE 2

AGE DISTRUBUTION

FIGURE 2

AGE DISTRUBUTION

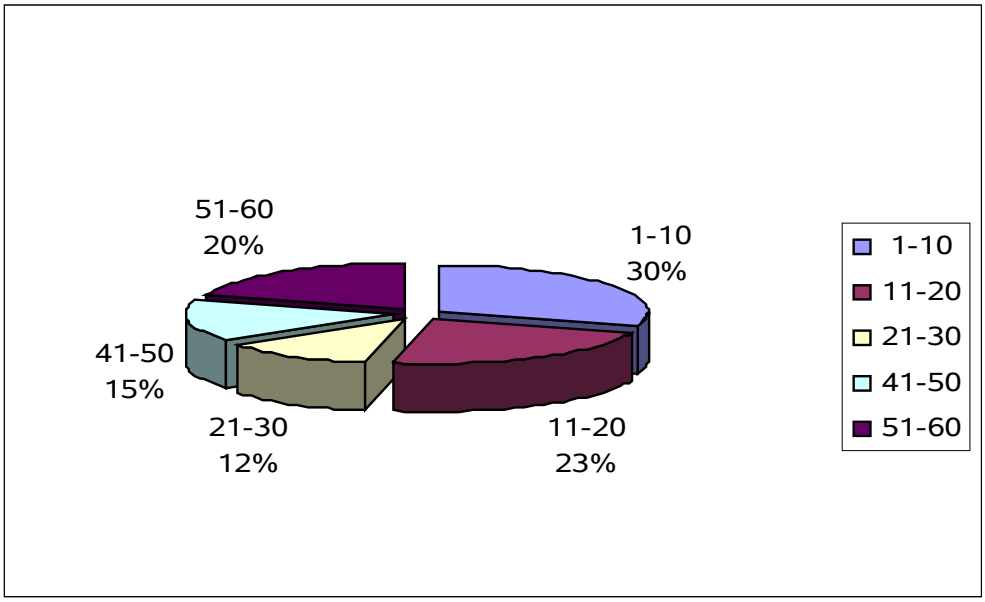


TABLE 3

DISEASES

No	Underlying disease	No of cases	HLA/GP positive
1	Acute leukemia	30	5
2	Chronic leukemia	4	1
3	Lymphoma	4	1
4	Aplastic anemia	2	1
	Total	40	8

Total of 40 serum samples were collected from patients who received multiple transfusion (minim with 10 units) over a period of 3-4 weeks. showed refractoriness (as

the platelet count did not increase as expected) the samples were tested 1) HLA and 2) GP iib/iiia epitopes antibodies by ELISA (GTI-MACE 1 kit).

Of these

- 8 patients were positive.
- Five (62.5%) patients were positive for HLA antibodies.
- Two (25%) were positive of GP.
- One (12.5%) patient was positive for both HLA and GP.

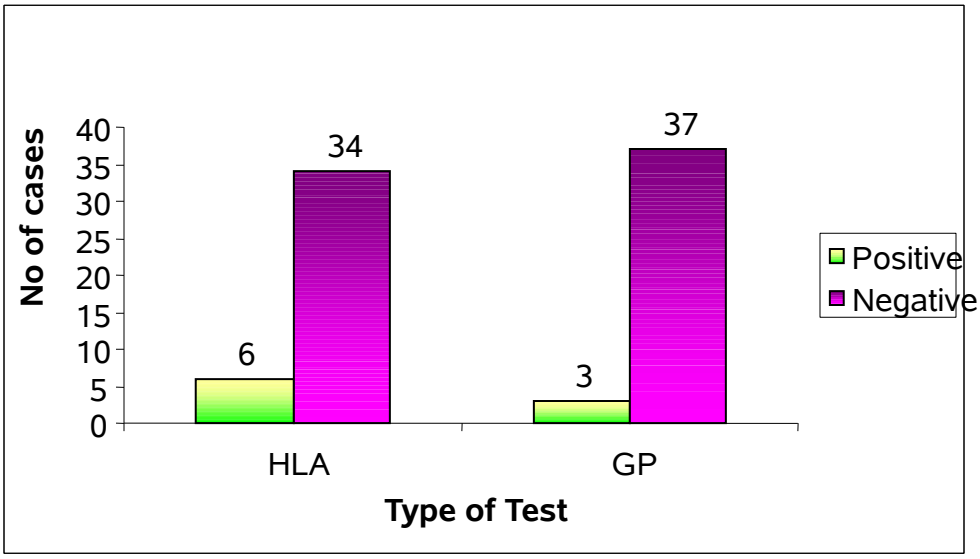
TABLE 4

HLA – GP COMPARETIVE

			GP		
			Positive	Negative	Total
HLA	Positive	Count	1	5	6
		% of Total	2.5%	12.5%	15.0%
	Negative	Count	2	32	34
		% of Total	5.0%	80.0%	85.0%
Total		Count	3	37	40
		% of Total	7.5%	92.5%	100.0%

FIGURE 3

HLA- GP COMPARETIVE



Sex wise:

Of these 8 patients, 4 were male (4/40) and 4 were female (4/15). HLA antibodies was positive in 3 male patients and 2 female patients, were as GP was positive in one of each male and female patients.

One female patient showed positive for both HLA and GP antibodies.

No of transfusion received:

- Total number of units transfused for each patient ranged from 10 to 36.
- 22 patients received 10-15 units of transfusion, of which 2 were positive for HLA and one for GP antibodies.
- 9 of them received between 15-20 units of which 2 were HLA positive and 1 GP antibodies were positive.
- 6 patients who received between 20-25 units showed one positive in both HLA and GP.

- One patient was positive to HLA antibodies in patients (3) who received more than 25 units.

TABLE 5

NO OF TRANSFUSION AND HLA

			HLA		
			Positive	Negative	Total
Total Transfusion	10 - 15 Units	Count	2	20	22
		% of Total	5.0%	50.0%	55.0%
	15 - 20 Units	Count	2	7	9
		% of Total	5.0%	17.5%	22.5%
	20 - 25 Units	Count	1	5	6
		% of Total	2.5%	12.5%	15.0%
	> 25 Units	Count	1	2	3
		% of Total	2.5%	5.0%	7.5%
Total		Count	6	34	40
		% of Total	15.0%	85.0%	100.0%

FIGURE 4

COMPARISION OF TRANSFUSION AND HLA

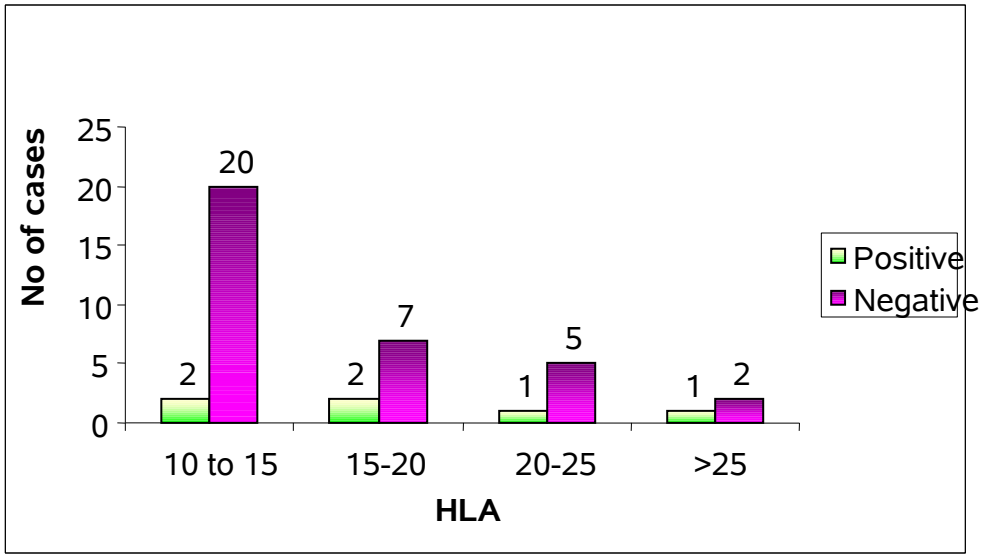


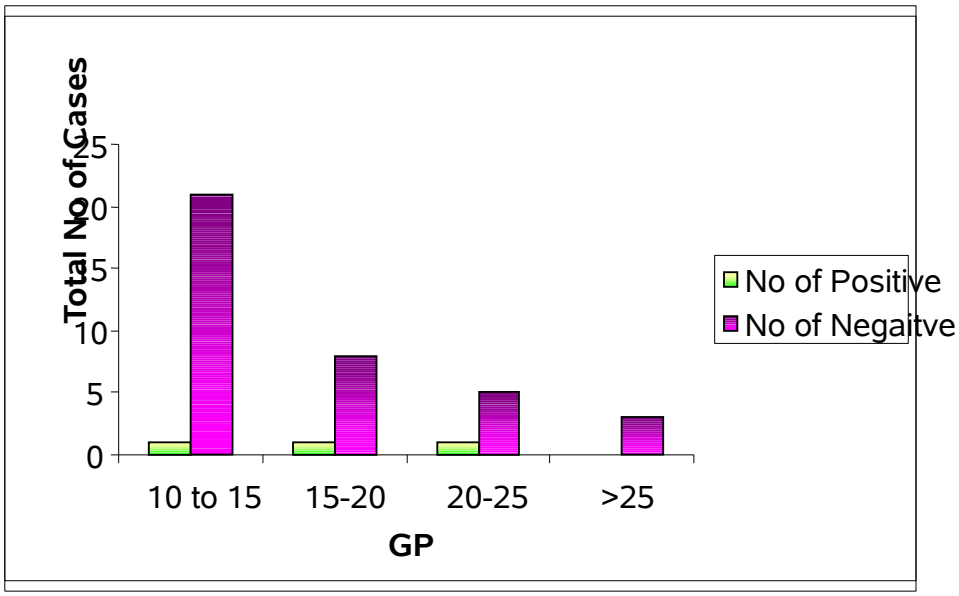
TABLE 6

NO OF TRANSFUSION AND GP

			GP		
			Positive	Negative	Total
Total Transfusion	10 - 15 Units	Count	1	21	22
		% of Total	2.5%	52.5%	55.0%
	15 - 20 Units	Count	1	8	9
		% of Total	2.5%	20.0%	22.5%
	20 - 25 Units	Count	1	5	6
		% of Total	2.5%	12.5%	15.0%
	> 25 Units	Count		3	3
		% of Total		7.5%	7.5%
Total		Count	3	37	40
		% of Total	7.5%	92.5%	100.0%

FIGURE 5

NO OF TRANSFUSION AND GP



Clinical history

Four female patients of 15 had history of single pregnancy in this. Two out of this study group showed positive (1 HLA and 1 GP).

15 patients had history of fever of which five were positive for antibodies (3HLA, 2GP).

Out of 40 patients 14 were anemic and four showed HLA and one GP antibodies.

Out of 40 patients 10 had symptom of jaundice. 4 of the jaundice patients showed positive for antibodies.

TABLE 7

SIGNS AND SYMPTOMS

NO	SIGNS/SYMPTOMS	POSITIVE FOR SIGNS /SYMPTOMS	HLA/GP POSITIVE
1	Fever	15	5
2	Anemia	14	5
3	Jaundice	10	4
4	Lympadenopathy	8	4
5	Splenomegaly	15	6
6	Pregnancy	4	2

DISCUSSION

The aim of this prospective study

a) **To evaluate the incidence of platelet alloimmunization in multiple transfused patients.**

b) **To compare frequency of HLA and GP antibodies.**

This study group consist of 40 patients out of which 8(20%) showed antibodies, In spite of the heterogeneity of patients population, treatment, and transfusion.. HLA antibodies were detected in 15% (6/40) of the patients and 7.5% (3/40) of patients were positive for GP iib/iiia antibodies who received multiple blood and platelet transfusion. This study confirmed that alloimmunization usually involves HLA antigens than platelet specific antigens.

The incidence of HLA alloimmunization in our study is in accordance with other studies. According to Taaning et al 1997 showed in their study that 15% of them who were antibodies negative before transfusion, turn out positive for HLA after multiple transfusion. Study conducted by Bajpai menu et al 2005; Kiefel v et al 2001 showed higher percentage (30%-70%) developed antibodies after multiple transfusion Platelet specific antibodies against Gpiib/iiia antigens were found to be Lowe than HLA in many studies. Kiefel v et al reported 45% patients had HLA whereas 8% of patients were HPA positive. Uhrynowska m et al showed that 21% had HLA antibodies and than 9% had HPA antibodies. Tanning et al 1997; Bertrand Godeau et al 1992 studies showed that platelet specific antibodies are even lower than 5%.These discrepancies could be due to the nature of test methods adopted, the immune status

of the patients, quality of platelets transfused and basic pathology in patients. In our study only one patient showed positive for both HLA and GP antibodies, in contrast to other studies which showed more no of cases with both positive. (Lo sc et al 2005 reported 22% had both HLA and HPA antibodies.)

Out of 8 patients, five had acute leukemias (ALL-2/23, AML-3/7) and one in each of other disorders (chronic leukemias-1/4, lymphomas –1/4, aplastic anaemia-1/2). In acute leukemias AML has higher frequency (40%) to that of ALL of 9% in accordance with study reported by Docoteau et al 1995. This difference is due to immunosuppression and altered immune status of the disease or immunosuppressive therapy. This may altered the response of platelet transfusion.

Women with the history of single pregnancy (4patients) showed a significantly higher incidence of alloimmunization (50%) as compared to patients with no such history. The result in this study is in accordance with the study done by Bajpai M et al 2005, states that 83% of the women with positive obstetric history had antibodies and 60% with negative history.

Fever was present in 3 of the patients who were positive for HLA antibodies and 2 GP iib/iiia antibodies positive patients in this study which were also reported by Masanoir shimoyama et al 1977. Jaundice and anaemia was seen in more than 50% of the patients who are positive for anyone antibody.

In this present study there was no definite association between the numbers of

transfusions received during the same admission with the development of antibodies. This study showed 3 patients who were positive after receiving transfusion between 10-15 units, 3 between 16-20 units, 1 patient between 21-25 units and 1 patient who received more than 25 units. This may be due to smaller number of cases or different donor-patient status.

Our study brings out a way for the early diagnose of platelet antibodies in multiple transfused patients, post transfusion Purpura and neonatal alloimmune thrombocytopenia. With suitable modifications, using newer and advance techniques, antibodies can also be detected in lower titration levels.

SUMMARY

Incidence of alloimmunization due to HLA class 1 antigens and GP iib/iiia epitopes in patients who were transfused with multiple blood and/or blood components had been studied and compared with other studies and were found to be less frequency than other studies.

HLA antibodies were found to be more than GP antibodies and female patients with positive pregnancy are more prone to development of refractoriness.

Increased incidence of alloimmunization is seen in AML than ALL.

There is no definite association between the numbers of units transfused and the formation of antibodies that leading to refractoriness.

CONCLUSION

Our results concluded that multiple random donor blood and platelet transfusion are able to induce antibodies against HLA class 1 antigens and epitopes on GP iib/iiia. There was no relationship between the number of units transfused and the antibodies formation. Women with single pregnancy history were identified as high responder group for alloimmunization. Our results show AML are frequently immunized than ALL patients.

Keeping in view the alloimmunization, leukoreduced irradiated single donor platelets and blood components should be transfused for the treatment of hematological and oncological patients. Till now, non-leukoreduced components are used in most of the centers in India due to cost of leukoreduction. Testing for the presence of platelet antibodies and transfusion of compatible platelets shall be important mode of management and prevention of platelet refractoriness in India.

LIMITATIONS OF THE STUDY

- The study period was limited to one year and the number of patients in the study was only 40.

- The duration of study is only one year. Long term follow up was not possible as many patients were outstationed.

- The samples were collected only from the oncology patients. .

- The study method could not be compared with the other methods to find the sensitivity and specificity.

FUTUROLOGY

The number of patients in the study can be increased and the patients other than oncological disorders can also be included in the study.

The study can be done for longer period and the patients can be followed upto 1 year by testing for antibodies at regular intervals even through transfusions are not given to check for disappearance of antibodies.

The study can be done more specifically by testing individually for antibodies against HLA class 1 A and B, HPA 1, 2, 3 and can also be done with other methods to find the best method to validate the test.

Female with the history of multiple pregnancies should be studied.

Patients who are receiving single donor platelet and the leukoreduced blood products alone can be included in the study to compare the incidence of alloimmunization and efficacy of leukoreduction.

This study can also be extended to find out the efficiency of management and prevention of alloimmunization by using HLA and HPA matched transfusion.

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ANNEXURES

PROFORMA

Study: PLATELET ALLOANTIBODY SPECIFICITIES IN MULTIPLY TRANSFUSED PATIENTS

Case ID: DIAGNOSIS:

Age/Sex:

Occupation:

Address:

Short clinical history:

COMPLAINTS	YES	NO
1) Gum bleed :		
2) Petechiae/purpura :		
3) Hematuria :		
4) Melena :		
5) Visual disturbance :		
6) Fever :		
7) Arthralgia :		
8) Others :		

Co morbid Diseases:

1) HT/DM/PT/CAD/BA if yes -

Treatment history:

1) Drug Intake ; if yes -
2) Surgery ; if yes -
3) Any other illness ; if yes -

Transfusion history:

Products	Date of Tx	Total no units
a) WHOLE BLOOD -		
b) PRBC -		
c) PLATELETS -		
d) PLASMA -		
e) OTHERS -		

Personal history:

1) Diet : veg non-veg

2) Smoking : yes no

3) Alcohol : yes no

Pregnancy history:

No of conceptions :

Date of Last delivery :

Family History:

1) Bleeding disorders : if yes

2) Malignances : if yes

3) Contact history : if yes

Clinical examination

1) Anemia : yes no

2) Jaundice :

2) Purpura : yes no

3) Petechia : yes no

4) Joint swelling; yes no

5) Focal infection: yes no

6) Lympadenopathy: yes no

7) Splenomegaly: yes no

Investigations already done:

DATE

1) Hb

2) TC

3) DC

4) ESR

5) Platelet count:

DATE OF PLATELET Tx pre Tx post Tx

6) PT

7) BT

8) CT

9) APTT

10) Blood grouping

11) Coomb's Test

12) Special investigations:

Patient Consent: