Alloantibody Assays and Outcome of Platelet Transfusions

Financial support for this thesis was granted by:
Schering, GlaxoSmithKline, Orthobiotech, Roche, Aventis and Pfizer

Cover: drawing desined bij Hans Kneefel, the platelets of the author by electronic microscopic view in the background
Printed by: Meuldijk & van Loon
ISBN: 90-9018871-1

Alloantibody Assays and Outcome of Platelet Transfusions

Alloantistoftesten en de opbrengst van trombocytentransfusies

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

donderdag 23 december 2004 om 13:30 uur
door
Mark-David Levin
geboren te Rotterdam.

Promotiecommissie

Promotor:
Prof.dr. B. Löwenberg

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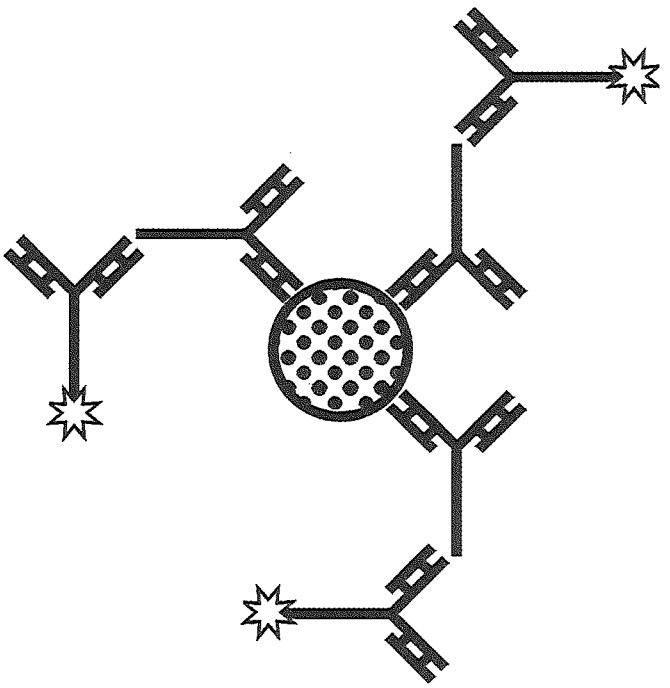
General introduction



Platelet transfusions

Patients treated for hematological illnesses frequently experience prolonged periods of thrombocytopenia. The causes of thrombocytopenia are numerous and can be multifactorial, such as: therapy related aplasia (chemotherapy, immunotherapie or radiation), the repression of healthy bone marrow by an underlying illness, primary bone marrow aplasia, graft failure after bone marrow transplantation and graft versus host disease. Severe thrombocytopenia can result in mild to life-threatening bleeding complications[1], which can be largely prevented by the timely application of donor platelet transfusions[2,3]. Prophylactic donor platelet transfusions often consist of a mixture of platelets from different random volunteer donors. They are administered when platelet count is less than 10 x 10^9/L[4-9] or at higher counts during interventions or in patients with an increased bleeding risk (e.g. concomitant administration of anticoagulant drugs and acute promyelocytic leukemia). The efficacy of platelet transfusions can be deducted from the increase in platelet count after transfusion. More exact methods of measuring the efficacy of platelet transfusions are platelet recovery, fractory increase of platelet count corrected by the blood volume, or corrected count increment (CCI), fractory increase of platelet count corrected by the body surface area[10-12]. In this thesis we used the platelet recovery as standardized measurement for efficacy of platelet transfusions and is defined as:

$$\% \text{ recovery} = \frac{\text{Platelet increment } (10^9/\text{L}) \text{ x blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \text{ x } 100$$

The platelet recovery is regarded as successful when ≥ 20% at 1 hour after platelet transfusion and ≥ 10% at 16 hours after platelet transfusion.

Platelet refractoriness

Twentyfour to 44% of random donor platelet transfusions fail to reach the desired platelet recovery after 1 or 16 hours[13-16], which is called a platelet transfusion failure. Platelet transfusion failures are caused by immune or non-immune factors interfering with the survival of transfused donor platelets *in vivo*. Immune causes of transfusion failure are alloantibodies directed against class I human leukocyte antigens (HLA-class I), the only HLA-antigens that are present on platelets, against platelet specific antigens (HPA), against antigens of the ABO-blood group system, autoantibodies

against platelets or circulating immune complexes[17-19]. Many non-immune factors have been described of which the most frequently encountered factors are: splenomegaly, fever, concomitant medication (e.g. antibiotics, fungizone, cyclosporine, tacrolimus, ciprofloxacin, vancomycin, heparin, ticlopidine), disseminated intravascular coagulation, radiotherapy and active blood loss. Regularly patients do not show any increase in platelet count inspite of repetitive random donor platelet transfusions resulting in platelet transfusion refractoriness and causing persistent severe thrombocytopenia[13–16,20-23]. It is important to prevent and, if possible, to eliminate the cause of the refractoriness. Therefore, alloantibody tests are used to detect the presence of immune causes of platelet refractoriness.

Prevention of alloimmunization

HLA-antigens are the most immunogenic antigens in platelet transfusions. These HLA-antigens are present not only on platelets but also on lymphocytes, monocytes and granulocytes. The presence of the latter cells in transfusion products (erytrocyte and platelet concentrates and fresh frozen plasma) play an important role in alloantibody formation in the patient. It is shown that the overall incidence of alloantibodies against HLA-class I antigens can be reduced by administrating leukodepleted blood products to patients[10,11,24,25-33]. Leukodepletion is obtained by applying commercially available white blood cell filters to all blood products, erythrocyte and platelet concentrates. Saarinen et al. demonstrated that filtrating blood products reduces refractoriness of patients against donor platelets from 6-93% to 0-24%[30]. Especially when filtered blood products contained no more than 5 x 10^6 leukocytes per transfused unit of blood refractoriness against random platelet transfusions was reduced to an average 3% of patients (Table 1). The patients who are demonstrated in Table 1 have not been prospectively studied according to similar in- and exclusion criteria. These data, however, are a summary of the available data that might give an estimation of the reduction in the prevalence of refractory patients to random donor platelet transfusions according to the amount of leukodepletion.

Table 1. Historical overview compiled from different studies: the number (percentage) of refractory patients in relation to the amount of leukodepletion

Refractoriness	Leukodepletion *n* (%)			*Total* *n*
	No [A]	Mild [B]	Rigorous [C]	
No	235 (76%)	473 (84%)	207 (97%)	915
Yes	96 (24%)	91 (16%)	7 (3%)	194
Total	331	564	214	1109

No leukodepletion means an average 64 – 1,060 x 10^6 leukocytes per unit blood, mild leukodepletion average 5-60 x 10^6 leukocytes per unit blood, and rigorous leukodepletion average < 5 x 10^6 leukocytes per unit blood

[A] *References 24, 25, 26, 28, 30, 31, 32, 33, 34*

[B] *References 24, 25, 26, 28, 31, 32*

[C] *References 30, 33, 34*

Transfusions in alloimmunized patients

The most frequently encountered alloantibodies in patients, refractory for random donor platelet transfusions who are treated for hematological illnesses, are directed against HLA-class I antigens[35]. HLA-class I matched single donor platelet transfusions (HLA-matched platelet transfusions) can overcome the refractoriness to transfused platelets. HLA-matched platelet transfusions are derived as a single donor apheresis product from volunteer donors who have been typed for their HLA-class I antigens. The HLA-matched platelet transfusions are classified as *compatible*, when donor and recipient share all HLA-A and B antigens or when the donor expresses less HLA-A and B antigens than the recipient (the donor is phenotypically homozygous for HLA-A and/or B loci). HLA-matched platelet transfusions are classified as *crossreacting*, when cross-reactive HLA-mismatches between the donor and recipient HLA-A or B antigens are present[36]. HLA-class C antigens have been thought to be of little significance in HLA-matching of transfused platelets[37]. Recently, however, a case series has demonstrated an improvement of the recovery of HLA-matched platelet transfusions if donor platelets are matched for the HLA-C locus too[38]. Further study is needed to clarify this issue.

Antibody detecting tests

In the past decades many different techniques that can detect alloantibodies against HLA-class I antigens have been developed (Table 2). All these techniques detect the presence of alloantibodies against HLA-class I antigens, but some techniques demonstrate other immune causes of enhanced platelet destruction too (platelet specific antibodies, autoantibodies or immune complexes).

Table 2. Different techniques to detect alloantibodies against HLA-class I antigens using donor panel cells

Test	Test principle	Type of antibodies	References
lymphocytotoxicity test (LCT)	Complement mediated lysis of donor lymphocytes	HLA	39 - 41
microscopic platelet suspension immunofluorescence test (PSIFT)	Microscopic detection of donor platelets with fluorescence labeled anti-IgG	HLA, platelet specific, autoantibodies, immune complexes	42
enzyme-linked immunosorbent assay (ELISA)	absorbance reading of donor platelets with enzyme labeled anti-IgG	HLA, platelet specific, autoantibodies, immune complexes	43 – 46
monoclonal antibody-specific immobilization of platelet antigens assay (MAIPA)	absorbance reading of lysated donor platelets with enzyme labeled anti-IgG after immobilization by a monoclonal antibody	Depending on the specificity of the monoclonal antibody HLA or HPA	47 – 49
monoclonal ^{125}I-labeled anti-IgG assay	radioactivity counting of donor platelets with ^{125}I-labeled anti-IgG	HLA, platelet specific, autoantibodies, immune complexes	50
flow cytometric platelet immunofluorescence test (PIFT)	flowcytometric detection of donor platelets with fluorescence labelled anti-IgG or anti-IgM	HLA, platelet specific, autoantibodies, immune complexes	51 - 56
flow cytometric lymphocyte immunofluorescence test (LIFT)	flowcytometric detection of donor lymphocytes with fluorescence labelled anti-IgG or anti-IgM	HLA	53 - 56
^{51}Cr platelet lysis assay	radioactivity counting of complement mediated lysis of ^{51}Cr-tagged donor platelets	HLA, platelet specific, autoantibodies, immune complexes	57
platelet radioactive antiglobulin test (PRAT)	radioactivity counting of donor platelets with ^{125}I-labeled anti-IgG after filtrating a membrane	HLA, platelet specific, autoantibodies, immune complexes	58

The value of alloantibodies detected by lymphocytotoxicity, flowcytometric immunofluorescence tests, MAIPA and ELISA has been previously assessed in relation to the survival of transfused platelets[13–16,20-23]. Only 3 of these studies were performed in patients receiving filtered blood products from random donors. The study by Ishida was confined only to patients undergoing hematopoietic stem cell transplantations; the study by Fabris registered only immune causes of refractoriness; and the study of Legler described refractory periods of patients, but not a correlation of test results with the recovery of a subsequent platelet transfusion.
Several new techniques have been introduced in recent years (Table 3). In these tests not panel cells are used as a target but HLA-antigens from a large volunteer donor group, which make these tests more standardized and more commercial.

Table 3. Different techniques to detect alloantibodies against HLA-class I antigens using standardized donor antigens

Test	Test principle	Type of antibodies	References
FlowPRA microbeads	flowcytometric detection of latex beads coated with a broad range of purified HLA-class I with fluorescence labeled anti-IgG	HLA	59, 60
QuikScreen™ (renamed ELIHLA in our studies)	absorbance reading of a broad range of solubilized HLA class I antigens with enzyme labeled anti-IgG	HLA	61 - 64

The value of these new techniques in relation the recovery of a subsequent leukodepleted platelet transfusion is not clear. Altogether, the contribution of immune and non-immune factors influencing platelet recovery in hematological patients receiving leukodepleted blood products still remains unclear despite previous studies.

Alloantibody detection in patients receiving leukodepleted blood products

Despite the application of leukodepletion to all blood products resulting in a reduction of the prevalence of immune-caused refractoriness to donor platelet transfusions, refractoriness by alloimmunization is still encountered (Table 1). The relative importance between antibodies against HLA-class I antigen, antibodies against platelet specific antigens (HPA) and non-immunological causes of platelet refractoriness have changed after the use of leukodepleted blood products[16]. It is not clear, however, what

percentage of platelet transfusion failures are caused by immunological or non-immunological factors in patients receiving leukodepleted blood products. When test characteristics of routinely used tests, e.g. LCT with a false positive result rate of 30%[13] as demonstrated in Table 4, are applied to a patient population which exhibits a diminished prevalence of alloantibodies against HLA-class I, because of the administration of leukodepleted blood products, it is to be expected that an increased rate of false positive test results will be encountered.

Table 4. Number of non-leukodepleted platelet transfusions exhibiting good or poor corrected count increments in relation to the results of the lymphocytotoxic test

Lymphocytotoxicity test	**Corrected count increment**		***Total***
	n		***n***
	Good	**Poor**	
negative	377	265	642
positive	69	161	230
Total	446	426	872

Corrected count increment (CCI): good when CCI ≥ 10 or poor when CCI < 10.
Lymphocytotoxicity test is positive when the alloantibody grade was more than 1 of 4.
This results in the following testcharacteristics: sensitivity 85% (377/446), specificity 38% (161/426), positive predictive value 70% (161/230) and negative predictive value 59% (377/642) according to Bishop[13].

In order to estimate the rate of false positive test results, according to the test characteristics of the LCT[13], in patients receiving random leukodepleted donor platelet transfusions, we combined the results of Table 1 and 4:

$$\text{False positive} = \frac{(1-\text{sensitivity}) \times \text{not refractory patients}}{(1-\text{sensitivity}) \times \text{not refractory patients} + \text{specificity} \times \text{refractory patients}}$$

False positivity = 0.15 x 207 / (0.15 x 207 + 0.38 x 7) = 0.91 = 91%

This figure of 91% is a high number and is only a theoretical deduction from the available data. This number is susceptable to selection bias in the studies demonstrated in Table 1 and to the number of transfusions per patient in Table 4. It is, however, very probable that the problem of false positivity will be large in clinics treating patients receiving leukodepleted platelet transfusions. The increased rate of false positive test

results may be lessened by (i) using a test that exhibits better test characteristics, increased sensitivity and specificity, or (ii) applying these tests to selected refractory patients at risk of immunological causes of platelet refractoriness.

Rationale of the thesis

In this thesis we deal with two aspects of alloantibody assays: (i) test characteristics and (ii) performance of tests in different patient populations. The first part of this thesis focuses on the technical aspects of antibody detection guided by the following questions:

1. Is a technique using standardized antigens (ELIHLA) as sensitive as techniques using panel cells (LCT, LIFT, PIFT) and are the results of these 4 different techniques related?

2. Are the results of a technique that detects IgG bound to transfused platelets *in vivo* (IVBI-PIFT) related to those of an *in vitro* technique using panel cells (crossmatch-PIFT) or using standardized platelet antigens (ELIHLA)?

3. Is binding of IgG to transfused platelets *in vivo* related to poor platelet recovery?

4. Can the visual scoring method of the IVBI-PIFT reliably be objectivated by a mathematical method of histogram subtraction?

The second part of the thesis deals with the predictive value of alloantibody tests on platelet recovery of random platelet transfusions in a non-selected patient population and of HLA-matched platelet transfusions in a heavely selected patient population. In some of the studies non-immunological factors jeopardizing the survival of platelets were taking into account too. These value of alloantibody assays were studied by the following questions:

1. What is the prevalence of immune and non-immune causes of platelet transfusion failures in a non-selected patient population?

2. Which alloantibody tests and what non-immune causes are best related to platelet transfusion failures in a non-selected patient population?

3. Are the results of alloantibody tests influenced by incubation factors (*in vitro* versus *in vivo*) and panel cell composition, and what is the impact of these two factors on the relation of test results with platelet recovery?

4. Are alloantibody tests predictive for the recovery of HLA-matched platelet transfusions in patients refractory for random platelet transfusions in the absence of non-immunological factors?

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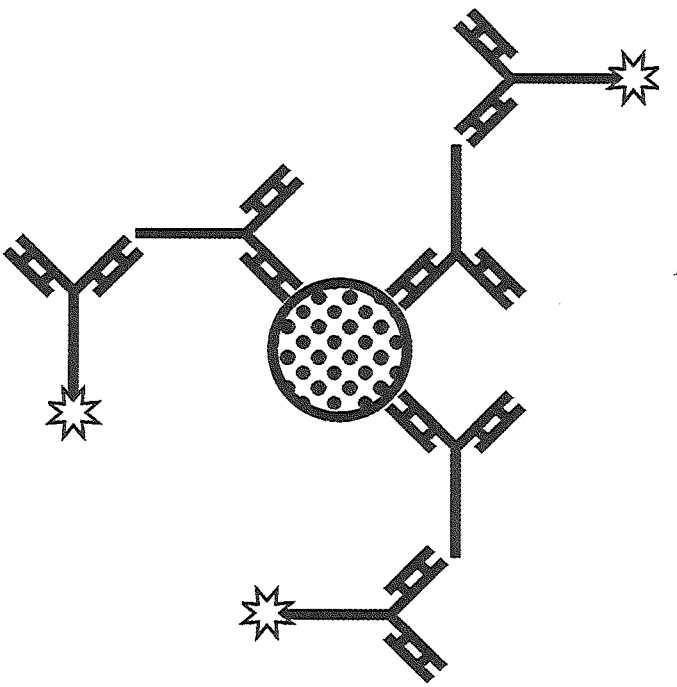
Chapter 2

Simultaneous detection of IgM and IgG antibodies against platelets, lymphocytes and granulocytes by flowcytometry



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Infusion Therapy and Transfusion Medicine 1998; 25: 317-324

Abstract

Background

Typically, immunofluorescence-based assays to detect antibodies against blood cells consist of separate tests on cell suspensions enriched for the individual populations. Here, we present a 3-color flow cytometric assay that allows the simultaneous detection of IgM and IgG antibodies against platelets, lymphocytes and granulocytes using a mixed suspension of these 3 cell populations.

Methods and materials

Platelets, lymphocytes and granulocytes are isolated and reconstituted in equal proportions, followed by incubation steps with serum and anti-human IgM and IgG conjugates. A mixture of laser dye solution 751 and propidiumiodide is added to the cells 10 min prior to flow cytometry to exclude residual erythrocytes and damaged and/or dead nucleated cells during data analysis on the basis of their nonreactivity, respectively very strong reactivity with these red-fluorescent dyes. Predefined, fixed light scatter and fluorescence (FL) region and marker settings, or definitions for cluster algorithms, are used in all experiments to identify the individual cell populations and to discriminate positive from negative immunofluorescence. This approach obviates the need for subjective adjustments of marker settings.

Results

We demonstrate the application of this assay using conventional list mode data analysis software (CellQuest™) and cluster analysis software (Attractors™). This flow cytometric assay is more sensitive than conventional assays (e.g., complement-dependent cytotoxicity or microscopic immunofluorescence assays) for the detection of alloantibodies against platelets, lymphocytes and granulocytes.

Conclusion

The simultaneous flow cytometric detection of IgM and IgG antibodies against platelets, lymphocytes and granulocytes is a rapid, sensitive and objective assay which is useful for detection of alloantibodies and crossmatching in transfusion medicine.

Introduction

The detection of antibodies against platelets, lymphocytes and granulocytes is important in hematological diagnosis and therapy. Demonstration of autoantibodies on platelets or granulocytes is helpful in establishing the autoimmune nature of thrombocytopenia and granulocytopenia[1]. Detection and specificity assessment of alloantibodies against platelets is crucial for the analysis of refractoriness to platelet transfusions. Antibodies against HLA antigens are reactive with platelets, lymphocytes and granulocytes, whilst platelet-specific antibodies react only with platelets. Serological crossmatching is useful for the selection of compatible donors for platelet transfusion therapy[2-4].

A wide variety of techniques has been developed to detect antibodies to platelets, lymphocytes and granulocytes[5-10]. During the last 2 decades the focus has been on antibody detection using conjugated anti-human antibodies. Radioactive isotopes, enzymes or fluorochromes have been used as tracer molecules in such assays. The assay presented here is based on immunofluorescence.

Direct and indirect immunofluorescence assays allow the detection of autoantibodies and alloantibodies, respectively. Direct assays include a single incubation step of cells with conjugated anti-human antibodies, followed by washing and reading. Indirect assays comprise 2 incubation steps: first, incubation of cells with patient (or control) serum, followed by washing; second, incubation with conjugated anti-human antibodies, followed by washing and reading. Such assays are typically performed on purified suspensions of platelets, lymphocytes and granulocytes. Microscopic immunofluorescence reading of cells in suspension requires that these suspensions are processed separately. These procedures are subjective and time-consuming.

With the use of flow cytometry there is no need for separate incubations of platelets, lymphocytes and granulocytes, as these cell types can be distinguished simultaneously on the basis of their forward (FSC) and sideward (SSC) light scatter characteristics in flow cytometric analyses. We have developed such an assay and present now several improvements in comparison to our previous report[11]. First, simultaneous detection of IgM and IgG antibodies is achieved by the use of a mixture of anti-human IgG and IgM antibodies labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively. Second, residual erythrocytes and damaged and/or dead nucleated cells are excluded from further analyses based on their nonreactivity, respectively very strong reactivity with a mixture of laser dye solution (LDS)-751 and propidiumiodide (PI). Third, we show the analysis of list mode data from this assay using automated cluster-defining software (Attractors™; Becton Dickinson [BDIS], San Jose, CA).

Methods and materials

Preparation of cell suspensions

Separate suspensions of platelets, mononuclear cells and granulocytes are prepared from a single EDTA-anticoagulated blood sample in order to prepare a test cell suspension in which the 3 cell populations are reconstituted in approximately equal proportions. This step is necessary to avoid the acquisition of too few list mode data of any of the 3 cell populations during flow cytometry. Platelet-rich plasma (PRP) is obtained by centrifugating the blood sample at 400 × *g* for 10 min. After removal of the PRP, residual platelets are harvested by reconstituting the cell pellet to the original volume of the blood sample using 0.027 M Na_2-EDTA in 0.013 M phosphate-buffered 0.145 M NaCl, pH 7.4, containing 0.2% bovine serum albumin (EDTA-PBS-BSA), centrifugation and collection of the supernatant. All centrifugation steps are carried out at room temperature (RT). The PRP and platelet-containing supernatant are pooled and centrifuged at 1200 × *g* for 20 min to obtain a platelet pellet, which is washed twice using EDTA-PBS-BSA. To obtain granulocyte and mononuclear cell suspensions, the cell pellet that remained after platelet removal is diluted to approximately its original volume by the addition of a 0.2% methylcellulose solution in EDTA-PBS-BSA, followed by incubation for 30 to 45 min at 37° C to allow gravity sedimentation of the erythrocytes. The leukocyte-containing supernatant is then harvested and the granulocytes are separated from the mononuclear cells by Ficoll density centrifugation at 1000 × *g* for 20 min. The interphase, containing the mononuclear cells, is collected and washed once with EDTA-PBS-BSA. Residual erythrocytes in the granulocyte pellet are lysed by precooled NH_4Cl solution for 5 min on melting ice, after which the granulocytes are washed once with EDTA-PBS-BSA. Thereafter, the mononuclear cells and the granulocytes each are washed once with EDTA-PBS without BSA (250 × *g* for 7 min) and fixed with 1% paraformaldehyde (PFA) in PBS for 5 min at RT, followed by 2 washes in EDTA-PBS-BSA. The platelets are then reconstituted to 150×10^9 cells/l and the mononuclear cells and granulocytes to 4.5×10^9 cell/l in EDTA-PBS-BSA. Finally, the test cell suspension is prepared by mixing equal volumes of the platelet, mononuclear cell and granulocyte suspensions. The use of mixed test cell suspensions yields similar results as obtained with separate suspensions of platelets, mononuclear cells and granulocytes[11]. Unstained platelets and mononuclear cells can be stored unmixed for up to 4 days for immunofluorescence assays to obtain similar results as with freshly isolated cells, whilst storage of granulocytes for >24 h results in increased background fluorescence.

Use of LDS-751 and propidiumiodide (PI) to exclude erythrocytes, damaged and dead nucleated cells from analysis

Residual erythrocytes may have similar FSC and SSC characteristics as lymphocytes and granulocytes (violet stained events in Figure 1). The presence of erythrocytes within the lymphocyte and granulocyte regions (see below) interferes with the exclusive detection of reactivity patterns of these cells and, therefore, will lead to erroneous results. LDS-751 stains DNA and RNA and hence allows discrimination between erythrocytes (negative) and nucleated cells (positive) on the basis of red fluorescence (FL). Damaged and dead nucleated cells, in particular granulocytes, may have increased autofluorescence and may bind antibodies nonspecifically. Thus, the presence of damaged and dead cells within the lymphocyte and granulocyte gates will hamper the sensitive and specific detection of antibodies on these cells. LDS-751 produces a signal of intermediate intensity in intact nucleated cells, but of very strong intensity in damaged cells[12]. Dead nucleated cells stain bright red with PI. Thus, the combined use of LDS-751 and PI allows the exclusion of erythrocytes on the basis of negativity, and the exclusion of damaged and dead cells on the basis of bright red immunofluorescence (Figure 1). A working mixture of LDS-751 and PI (LDS-PI) is prepared for each assay by diluting 5 µl of PI (Sigma, St. Louis, MO) stock solution (4 mg/ml in PBS) and 5 µl LDS-751 (Exciton, Dayton, OH) stock solution (0.2 mg/ml in methanol) in 10 ml EDTA-PBS-BSA.

Direct and indirect immunofluorescence assays

All incubations are performed in duplicate.

Direct assay:

50 µl of the test cell suspension are incubated with 50 µl of a mixture of equal volumes of goat-anti-human IgG conjugated with fluorescein isothiocyanate (GAHu/IgG/FITC; Nordic, Tilburg, the Netherlands) and goat-anti-human IgM conjugated with phycoerythrin (GAHu/IgM/PE; Southern Biotechnology Associates, Birmingham, AL) for 30 min in the dark at RT. The conjugated anti-human antibodies have been selected for their exclusive reactivity with human IgG and IgM, respectively, and titrated so as to obtain maximum discrimination between positive and negative signals. Following incubation the cells are washed three times using EDTA-PBS-BSA (250 × *g* for 7 min). After the last centrifugation step, the supernatant is removed so as to leave 25 µl buffer on the cell pellet. Following addition of 10 µl LDS-PI mixture, the cells are resuspended and incubated for 10 min prior to flow cytometry.

Indirect assay:

50 µl serum are incubated with 50 µl test cell suspension for 30 min at RT, followed by 3 washing steps. The cells are then incubated with 50 µl of the conjugate mixture (see

above) for another 30 min in the dark at RT. The cells are then washed and incubated with LDS-PI as described above.

Serum samples are harvested from whole blood that has been allowed to clot for 2 h at RT and stored at -70° C. A serum pool from multitransfused patients, reactive with platelets, lymphocytes and granulocytes from >90% of randomly selected cell donors, serves as positive control. Negative control sera are selected from sera obtained from untransfused males. The test cell suspensions must always chosen to be ABO compatible with the control and test sera, as anti-A and anti-B antibodies may react with platelets. This compatibility is easily realized by selecting only bloodgroup 0 donors for the cell panel. However, ABO-incompatible platelet crossmatches may be required for the selection of HLA-matched, ABO-incompatible platelet donors. The possible contribution of anti-A and anti-B antibodies to positive crossmatches can be assessed in such cases by including in the assay a patient serum sample from which the anti-A and/or anti-B antibodies have been removed by adsorption.

Flow cytometry

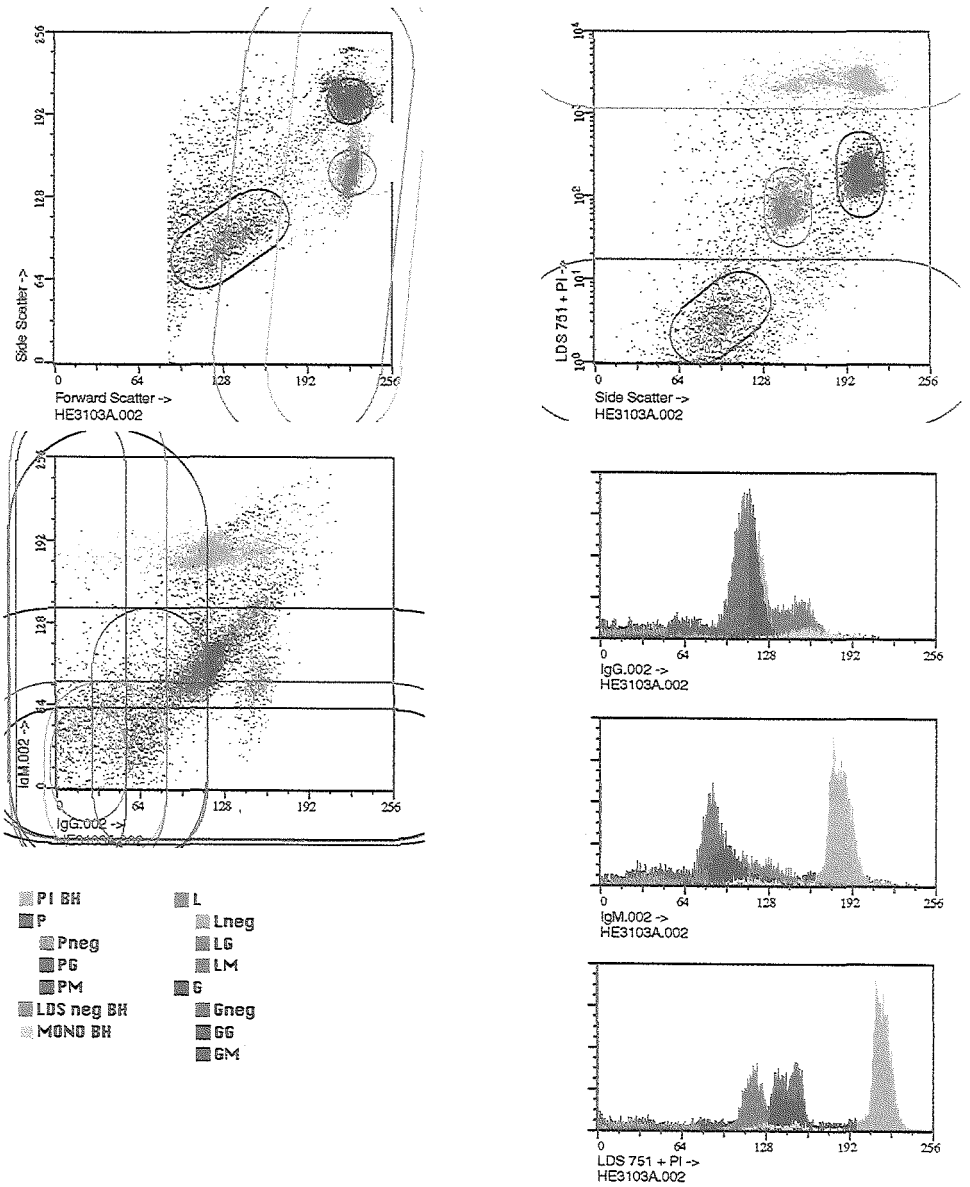
Data acquisition:

We perform flow cytometry on a FACScan instrument connected with a Macintosh Quadra 650 computer equipped with CellQuest™ and Attractors™ software (BDIS). The appropriate instrument settings (FSC photodiode, SSC, FL1, FL2, FL3 photomultiplier tubes, FL1 _ FL2 and FL2 _ FL3 color compensation) are verified prior to each experiment using QCWindows™ microbeads (Flow Cytometry Standards Corporation, San Juan, PR) and lymphocytes that are either unstained or stained with CD4/FITC or CD8/PE[13]. Careful instrument calibration is important in view of the use of predefined, fixed region and marker, or attractor, settings in all experiments (see below). Logarithmic scales are used for all parameters. Five-parameter (FSC, SSC, FL1[FITC], FL2[PE] and FL3[LDS/PI] list mode data of a minimum of 2,000 events per cell population, but at least 8,000 events, are acquired using CellQuest™ software.

Data analysis using CellQuest™ software:

Platelets, lymphocytes and granulocytes are selected by setting fixed regions in FSC vs. SSC, and SSC vs. FL3 dot plots. Platelets are FSC^{low}, SSC^{low} and $FL3^{low}$ (green); lymphocytes are FSC^{high},$SSC^{intermediate}$ and $FL3^{intermediate}$ (red), whilst granulocytes are FSC^{high}, SSC^{high} and $FL3^{intermediate}$ (blue). Using CellQuest™, regions are placed similarly as the green, red and blue attractors in Figure 1 (upper 2 panels).

Figure 1. Analysis of a serum containing IgM antibodies against platelets and lymphocytes, and IgG antibodies against platelets, lymphocytes and granulocytes using Attractors™ software (BDIS)

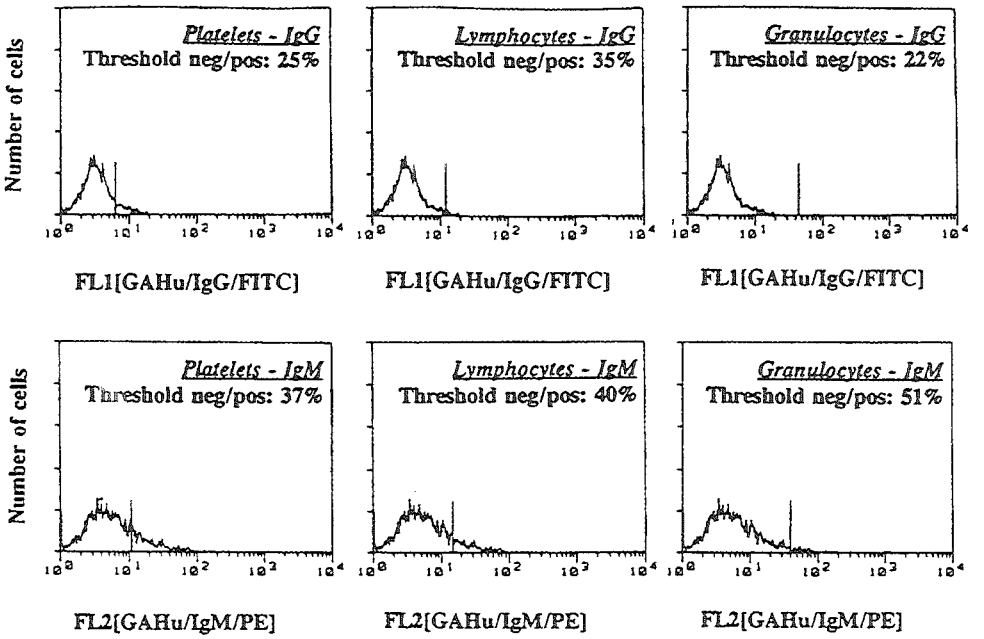


Events are assigned to cell types by a set of hierarchical attractors. First, dead and damaged cells are excluded from further analyses by defining a 'black hole' attractor on the basis of strong FL3[LDS-751 + PI] positivity (orange), placed at a fixed distance from the lymphocyte attractor

(FL3^{intermediate}). Second, platelets are FSC^{low}, SSC^{low} and FL3^{low} (green). Third, residual erythrocytes are excluded by their definition as remaining FL3 negative events (violet) by placing a 'black hole' attractor at a fixed distance from the lymphocyte attractor (FL3^{intermediate}). Fourth, monocytes are excluded on the basis of their FSC and SSC characteristics (yellow) by placing a 'black hole' attractor at a fixed FSC,SSC distance from the lymphocyte attractor. Fifth, lymphocytes are FSC^{high},SSC^{intermediate} and FL3^{intermediate} (red). Sixth, granulocytes are FSC^{high}, SSC^{high} and FL3^{intermediate} (blue). The placement of 'black hole' attractors at fixed distances of flexible attractors was done to circumvent the inability of the software to place flexible attractors on clusters containing too few events. The binding of human IgG (FL1[FITC]) and IgM (FL2[PE]) antibodies is analyzed using fixed attractors. These have been defined for negative serum reactions with each cell population (Pneg, Lneg and Gneg), whilst another set of attractors encompasses the full range of IgG/FITC immunofluorescence (PG, LG and GG) and a third set the whole range of IgM/PE immunofluorescence (PM, LM and GM). The percent cells with positive immunofluorescence for each cell type and Ig class is defined as the percent of events in the 'full FL range' attractor minus the percent of events in the corresponding 'negative population' attractor. Note that the damaged and dead cells (orange) stain strongly with GAHu/IgM/PE (middle histogram), whilst the monocytes (yellow) react positively with GAHu/IgG/FITC (upper histogram).

Fixed regions can be used throughout each experiment regardless of the number and type of cell donors (i.e., patient vs. blood bank donor) tested. FL1 (GAHu/IgG/FITC) and FL2 (GAHu/IgM/PE) immunofluorescence are analyzed using histograms and predefined marker settings that are kept constant throughout each experiment *and* between different experiments. These marker settings (Figure 2) have been defined for each cell population (i.e., platelets, lymphocytes and granulocytes), each anti-human Ig conjugate (i.e., IgM and IgG) and each assay type (direct and indirect immunofluorescence; see Results).

Figure 2. Predefined FL marker settings for platelets (left panels), lymphocytes (middle panels) and granulocytes (right panels), for the detection of antibodies of the IgG class (upper panels) and antibodies of the IgM class (lower panels)



The threshold levels for the classification of serum reactivity as positive or negative are shown in each histogram.

Data analysis using Attractors™ software:

The analysis is based on the definition of a hierarchical set of attractors, i.e., once events have been clustered by one attractor they cannot be counted by subsequent attractors. Events are assigned to cell types by a set of flexible attractors. First, dead and damaged cells are excluded from further analyses by defining a 'black hole' attractor on the basis of strong FL3[LDS-751 + PI] positivity (orange events in Figure 1). Second, platelets are FSC^{low}, SSC^{low} and $FL3^{low}$ (green events). Third, residual erythrocytes (violet events) are excluded by their definition as remaining FL3 negative events. Fourth, monocytes are excluded on the basis of their FSC characteristics (higher than lymphocytes and granulocytes) and SSC characteristics (intermediate between lymphocytes and granulocytes; yellow events), in order to avoid their binding of human IgG antibodies through their high-affinity Type I Fc(IgG) receptors[14]. Fifth, lymphocytes are FSC^{high}, $SSC^{intermediate}$ and $FL3^{intermediate}$ (red). Sixth, granulocytes are FSC^{high}, SSC^{high} and $FL3^{intermediate}$ (blue).

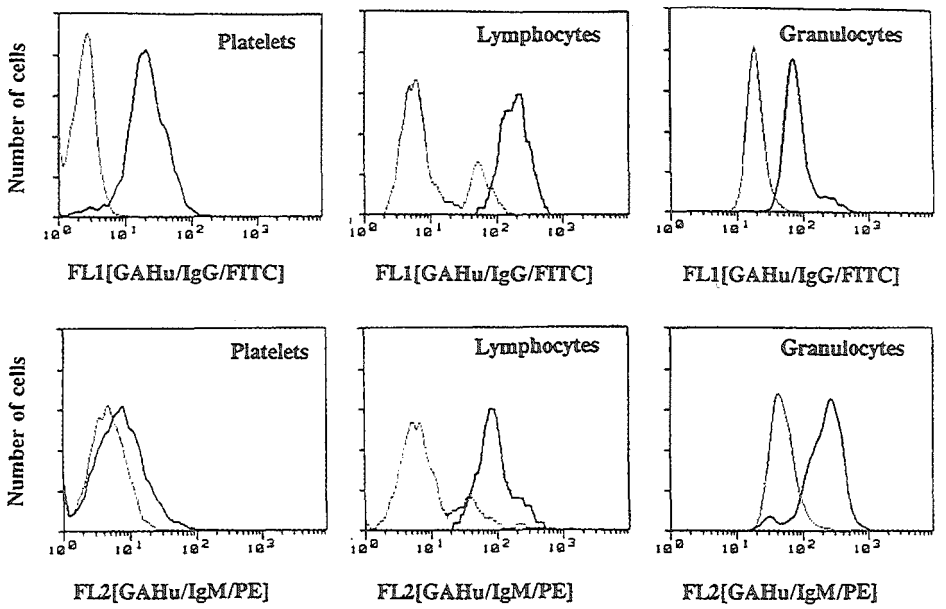
The binding of human IgG (FL1[FITC]) and IgM (FL2[PE]) antibodies is then analyzed as follows. Fixed attractors have been defined on the clusters defined by FL1 and FL2 using 200 list mode data files ('negative' sera from 20 untransfused male donors tested against cells from 10 blood group 0 donors) in such a way that similar results were obtained as with conventional marker setting (see Results). These attractors have been defined for platelets, lymphocytes and granulocytes separately (Pneg, Lneg and Gneg in Figure 1). Another set of attractors encompasses the full range of IgG/FITC immunofluorescence (PG, LG and GG) and a third set the whole range of IgM/PE immunofluorescence (PM, LM and GM). The percent cells with positive immunofluorescence for each cell type and Ig class is defined as the percent of events in the 'full FL range' attractor minus the percent of events in the corresponding 'negative population' attractor.

Results

Definition of FL1 and FL2 marker settings for indirect immunofluorescence assays

To define these markers to discriminate between positive and negative immunofluorescence in the indirect assays, cells from 10 blood bank donors (bloodgroup 0) were incubated with 20 sera from untransfused male blood bank donors (i.e., 'negative' sera) and the positive control serum. Examples of the reactivities of negative and positive control sera with platelets, lymphocytes and granulocytes as visualized with GAHu/IgG/-FITC and GAHu/IgM/PE, are shown in Figure 3.

Figure 3. Examples of the reactivities of negative (interrupted lines) and positive (continuous lines) control sera with platelets, lymphocytes and granulocytes as visualized with GAHu/IgG/FITC (upper panels) and GAHu/IgM/PE (lower panels)



The resulting list mode data were analyzed using a series of marker settings. For each cell donor, cell population and marker setting the average of the percentages of positive cells after incubation with each of the 20 negative sera was calculated and subtracted from the corresponding percentage of positive cells after incubation with the positive control serum. The marker setting yielding the largest difference between these 2 values was then selected for each donor; from the 10 marker settings thus obtained, the median was chosen as the predefined marker setting for all subsequent experiments.

A threshold level of percent positive cells was then defined to classify the reactivity of a given serum with a given cell population from a given donor as detected by a given anti-human Ig conjugate as positive or negative. For each cell type and anti-human Ig conjugate, the 98th percentile of the percentage of positive cells obtained with the predefined marker setting on the list mode data of the 200 data points (20 sera × 10 cell donors) was assessed (Figure 3). Sera yielding percentages of cells exceeding that threshold are classified as positive for that cell population, that donor and that anti-human Ig conjugate.

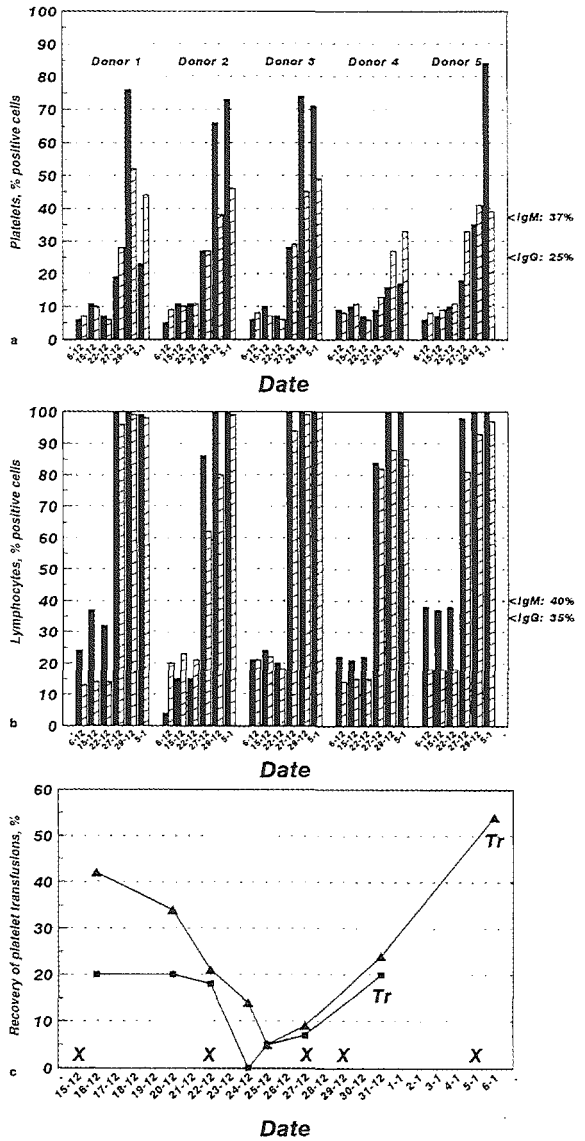
Performance and application

The flow cytometric detection of alloantibodies against platelets and lymphocytes is more sensitive than detection by immunofluorescence microscopy, whilst both detection methods perform similarly in the detection of antibodies against granulocytes[11]. Expectedly, our flow cytometric assay (LIFT) is also more sensitive than the complement-cytotoxicity assay (LCT) in detecting lymphocyte-reactive antibodies, because the LCT does not detect antibodies that do not bind complement. Of 26 sera that were positive in the LIFT, only 12 were also positive in the LCT, whilst 1 of 26 sera that were negative in the LIFT, was positive in the LCT.

The hands-on time for a series of 16 sera (plus 1 positive and 1 negative control serum) tested against a panel of 5 donors in duplicate is 3 h for preparation of cell suspensions, 3 h for serum and conjugate incubations, 2 h for flow cytometric data acquisition and 1 h for processing the results of the automated flow cytometric data analysis.

We have investigated the occurrence of IgM antibodies against platelets, lymphocytes and granulocytes in a longitudinal study of 13 patients which received platelet transfusions during and after chemotherapy for hematological malignancies and which developed IgG anti-HLA antibodies. Both classes of such antibodies could be detected in all patients. An example is shown in Figure 4. A 63-year old male received chemotherapy for refractory anemia with excess of blasts in transformation (RAEB-t). On 27/12 strongly reactive IgM and IgG antibodies against lymphocytes from all of 5 panel donors became detectable, followed on 29/12 by IgM and IgG antibodies against platelets, indicating allosensitization against HLA. At that time, 1-hour and 16-hour recoveries of random donor platelet transfusions had dropped to almost nil. Administration of HLA-matched platelet transfusions on 31/12 and 6/1 led to improved recoveries.

Figure 4. A longitudinal study of IgM (hatched [right-hand] bars) and IgG (filled [left-hand] bars) class antibodies against platelets (upper panel) and lymphocytes (middle panel) in relation to 1-hour (⩓) and 16-hour (≡) recoveries of platelet transfusions (lower panel)



The thresholds between positive and negative scores for IgM and IgG immunofluorescence on platelets and lymphocytes are shown at the right hand of the figure. X signs indicate the dates on which alloantibody studies were performed. Tr, HLA-matched platelet transfusion. For explanation, see Results.

Discussion

The standardized isolation and staining procedures of the cells and the careful calibration of the flow cytometer settings allow, with conventional data analysis software (e.g., CellQuest™), the use of fixed region and marker settings throughout an experiment, no matter how large. The use of software using cluster analysis (i.e., Attractors™) has the advantage that the flexible attractors defining the cell types will move along with small variations in light scatter characteristics and FL3 staining of the cells. This is advantageous in the case of LDS-751 which shows reversible binding to DNA/RNA as a function of dye concentration in the cell suspension[12]. This situation permits time-saving, walk-away, fully automated analysis of the list mode data. The results of these analyses can be transmitted electronically to a PC environment and processed to clinical reports using spreadsheets. In addition, we have developed an interface allowing the connection of the flow cytometry unit to our hospital information system LABZIS-2 (Hiscom, Leiden, The Netherlands).

The combined detection of alloantibodies against platelets, lymphocytes and granulocytes provides a good indication of the specificities of such antibodies (Table 1).

Table 1. Simultaneous detection of antibodies against platelets, lymphocytes and granulocytes: examples of interpretation of results

Cell population	Cell donor					Interpretation
	1	2	3	4	5	
Platelets	+	+	+	+	+	Platelet-specific antibodies
Lymphocytes	-	-	-	-	-	
Granulocytes	-	-	-	-	-	
Platelets	+	+	+	-	-	anti-HLA antibodies
Lymphocytes	+	+	+	-	-	
Granulocytes	+	+	+	-	-	
Platelets	+	+	+	+	+	anti-HLA and platelet-specific
Lymphocytes	+	+	+	-	-	antibodies
Granulocytes	+	+	+	-	-	

+ or -, Sera yielding percentages positive cells exceeding that threshold are classified as positive for that cell population, that donor and that anti-human Ig conjugate.

However, a reaction pattern exclusively with platelets may also be caused by antibodies against so-called cryptantigens. Such antigens are normally hidden in the cell membrane, but are exposed as a result of interaction between EDTA, PFA or a combination of both

compounds with the platelet membrane[15]. The binding of calcium ions by EDTA results in a change in conformation of the platelet glycoprotein IIb/IIIa complex. The ability of PFA to interact in such a way with platelets has led us to abandon fixation of platelets in PFA (see above). EDTA-dependent, platelet-specific antibodies are clinically not relevant. Additional assays with platelets derived from citrate-anticoagulated blood are required as such antibodies hamper the interpretation of the test results.

In conclusion, we have shown that using three-color flow cytometry, IgM and IgG antibodies against platelets, lymphocytes and granulocytes can be detected simultaneously in a sensitive, rapid and standardized assay of which data analysis and processing can be automated to a high degree.

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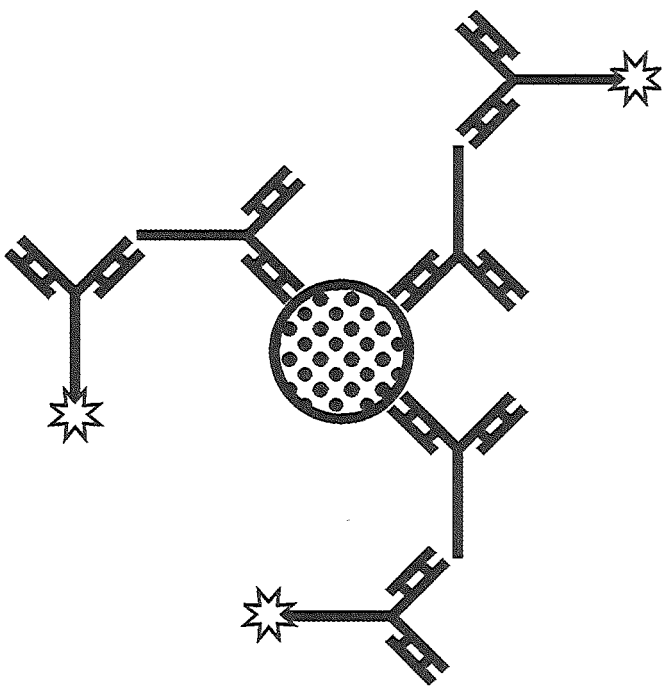
Chapter 3

Screening for alloantibodies in the serum of patients receiving platelet transfusions: a comparison of the ELISA, lymphocytotoxicity and the indirect immunofluorescence technique



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Transfusion 2003; 43: 72-77

Abstract

Background

For screening of alloimmunization in patients repeatedly receiving platelet transfusions different tests are used of which none is the standard. Here we describe a comparison of four tests most commonly used for detection of allo-immunization in a group of non-selected patients receiving platelet transfusions.

Methods and materials

In 99 patients with hematological malignancies who received platelet transfusions 192 random serum samples were tested in the Enzyme-Linked Immunosorbent Assay (ELISA), the Lymphocytotoxic Test (LCT), the Lymphocyte Immunofluorescence Test (LIFT) and the Platelet Immunofluorescence Test (PIFT). Results of all tests were mutually compared.

Results

The results of all tests were significantly correlated with each other ($P < 0.005$). ELISA and LIFT were more often positive than LCT and PIFT. ELISA and LIFT showed the best correlation ($\chi^2 = 63.7$; $P < 0.001$).

Conclusion

ELISA, the least time consuming test, detects alloimmunization as often as LIFT and more often than LCT and PIFT.

Introduction

Patients with hematological malignancies who receive intensified chemotherapy often need platelet support to prevent bleeding complications. Twenty-four to 44% of platelet transfusions fail to produce satisfactory response because of non-immune factors (63-67%), immune factors (18-25%) or both[1-4]. In order to distinguish immune from non-immune factors different serological tests are routinely used. In the past decades the Lymphocytotoxic Test (LCT)[5-7], microscopic Platelet Suspension Immunofluorescence Test (PSIFT)[8], Enzyme - Linked Immunosorbent Assay (ELISA)[9-12], Monoclonal Antibody - specific Immobilization of Platelet antigens Assay (MAIPA)[13-15], monoclonal ^{125}I-labeled anti-IgG assay (^{125}I-MA)[16], flowcytometric Platelet and Lymphocyte Immunofluorescence Test (PIFT respectively LIFT)[17-22], ^{51}Cr platelet lysis assay[23] and Platelet Radioactive Antiglobulin Test (PRAT)[24] have been described. In recent years several new techniques have been introduced such as flowcytometry of latex beads coated with HLA class I antigens[25,26], solubilized HLA class I antigens[27] and commercially available ELISA tests with a broad range of HLA class I antigens[28-30]. Here we compare the test results obtained with a commercially available ELISA with a large panel of HLA class I antigens with those of the LCT and LIFT. In order to detect anti-platelet antibodies against other than HLA Class I antigens the PIFT was also performed in these serum samples.

Methods and materials

Patients

From October 29th 1998 until August 1st 2000 192 serum samples from 99 random patients (Table 1), mean 1.9 transfusions per patient (range 1-8), who were treated for a malignant lymphoma or leukemia in our hospital were tested. Only one patient was treated with antithymocyte globulins; none with intravenous immunoglobulins. The serum samples were collected before the patients received a leucocyte depleted platelet transfusion, consisting of 5 random ABO-matched donors, prophylacticly when platelet count was less than 10 x 10^9/l, before an intervention or because of clinical manifest bleeding at higher platelet counts. The median platelet count before platelet transfusion was 11 x 10^9/l (range 4-60 x 10^9/l). Of 192 serum samples the ELISA failed in 6 samples and LCT failed in 3 samples because of technical reasons.

Table 1. Patient characteristics

Characteristics	Category	Results
Gender	Male	62
	Female	37
Age in years	Median	46
	Range	16-76
Diagnosis	ALL	5
	AML	43
	CLL	1
	CML	9
	MM	6
	NHL	18
	HL	4
	MDS	10
	Other	3
Therapy	Chemotherapy	61
	Auto BMT/SCT	10
	Allo BMT/SCT	23
	Other	5

ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphoblastic leukemia; CML = chronic myeloid leukemia; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; HD = Hodgkin's disease; MDS = myelodysplastic syndromes; Auto BMT/SCT = autologous bone marrow transplantation or peripheral stem cell transplantation; Allo BMT/SCT = allogeneic bone marrow transplantation or peripheral stem cell transplantation

ELISA

QuikScreen™ was used according to manufacturer's instruction (GTI, Brookfield, WI, USA) by incubating patient's serum with HLA class I antigens obtained from a large pool of platelets from white, black and hispanic blood donors immobilized in a microwell strip. After adding alkaline phosphatase conjugated goat-antihuman-immunoglobulin G (IgG) and a substrate of alkaline phosphatase p-nitrophenyl phosphate (PNPP) the absorbance was read at 405 nm. A result was considered to be positive when absorbance exceeded the absorbance of the negative control more than 2 times. A result was rejected when duplo results differed more than 20%.

LCT

Five ml of citrate whole blood was incubated with 1-2 g Fe^{2+} and 2.5 ml 135 mmol/l NaCl, 9.0 mmol/l $Na_2HPO_4.2H_2O$, 2.6 mmol/l $NaH_2PO_4.2H_2O$, 0.05% NaN_3 titrated up to pH = 7.2 (PBS) and was incubated for 30 minutes at 37 ^{0}C. After cooling for 10 minutes 2.5 ml of Lymphoprep (Pocas, Oslo, Norway) was injected beneath the suspension and centrifuged at 1000 G (Rotixa RP 4200, Hettich, Germany) for 20 minutes. The interfase was washed 2 times with 11 ml 1% bovine serum albumin in PBS (1% BSA-PBS) and centrifuged at 450 G for 5 minutes. The cell pellet was suspended in 1 ml RPMI1640/10% BCS (Bio Whittaker, Verviers, Belgium). Covered by a layer of Medinol oil 1 µl of donor leukocyte suspension with a concentration of 3 x 10^6/ml was incubated with 1 µl of patient serum for 30 minutes at room temperature and then 5 µl of rabbit complement was added to each well separate for 5 different donors. After incubating for 45 minutes 2 µl of eosin was added for 3 minutes after which 10 µl of formaldehyde was added. The wells were covered and left over night at 4 ^{0}C . The next day dead cells were judged by phase contrast microscopy in triplet. If 2 or 3 of the triplet showed a more than 50% lysis[7] the reaction against the donor was considered to be positive. If 2 or more of 5 donors showed a positive reaction the patient was considered immunized against a broad range of HLA-antigens.

PIFT and LIFT

In order to obtain platelet rich plasma (PRP) 10 ml donor EDTA anticoagulated blood was centrifuged 10 minutes with 400 G. PRP was reincubated with a suspension of 10 g ethyleendiamine-tetra-acetaat, 8.5 g NaCl, 1.61 g $Na_2HPO_4.2H_2O$ and 0.2 g $NaH_2PO_4.2H_2O$ in 1 liter Millipore H_2O, titrated up to pH = 7.35 with 10 mmol NaOH and mixed with 9 ml bovine serum albumin 22%, (EDTA-PBS 0.2% BSA) to a total of 10 ml and centrifuged at 1800 G for 10 minutes. After removing the supernatant platelets were resuspended to 7 ml with EDTA-PBS 0.2% BSA and centrifuged at 1800G for 10 minutes. After removing the supernatant platelets were resuspended with EDTA-PBS 0.2% BSA to reach a concentration of 100 x 10^6 per ml. To prepare a leukocyte cell suspension the suspension that remained after removal of PRP was resuspended to the original amount and lysated with precooled NH_4Cl during 20 minutes on ice and than centrifuged at 250 G for 10 minutes. After removing supernatant leukocytes were washed twice with EDTA-PBS 0.2% BSA at 250 G for 10 minutes and brought to a concentration of 6 x 10^6/ml with EDTA-PBS 0.2% BSA. Finally a mixed cell suspension was prepared by mixing equal volumes of platelet and leukocyte suspensions.

To 50 µl of mixed cell suspension 50 µl of patient serum was added. After incubation for 30 minutes in the dark the suspension was three times resuspended with 850 µl

EDTA-PBS 0.2% BSA and centrifuged at 250 G for 7 minutes and incubated with 50 µl diluted mixture of fluorescence-conjugated goat anti-IgG (FITC) (Nordic, Tilburg, The Netherlands). After incubation for 30 minutes in the dark the suspension was three times resuspended with 850 µl EDTA-PBS 0.2% BSA and centrifuged at 250 G for 7 minutes. Finally 10 µl LDS-PI (5 µl LDS stock (0.2 mg LDS 751 in 1 ml Methanol) (Applied Laser Technology, Maarheze, The Netherlands) together with 5 µl PI stock (4 mg propidium iodide in 1 ml PBS) (Sigma Company, St Louis, MO, USA) in 10 ml EDTA-PBS 0.2% BSA) was added to the suspension and incubated for at least 15 minutes.

Four parameter flowcytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San José, CA) equipped with an air-cooled argon laser emitting 488 nm at 15 mW. Forward light scatter (FSC) was detected by a solid-state silicon detector, sideways light scatter (SSC) by one photomultiplier tube, FITC by a second photomultiplier tube and a 530 nm bandpass filter, Laser Dye Solution (LDS-751) by a third photomultiplier tube and a >650 nm bandpass filter. During the analysis platelets and lymphocytes were discriminated by their FSC and SSC characteristics, while LDS-PI was used to diminish background interference of DNA and RNA remnants[31]. Data were analyzed by a Macintosh G4 (Apple, Cupertino, CA, USA) in order to determine which percentage of total platelet or lymphocyte population exceeded a specific marker channel of fluorescence. A donor was considered to be positive if more than 17% and 36% of all platelets (PIFT) and leucocytes (LIFT) exceeded a specific marker for each cell population, respectively. These percentages resulted from the data of over 100 negative controls. A result per donor was rejected when duplo results differed more than 10%. The percentage positivity of all donors, thus less than 5 if a result of one donor was rejected, was reported. A patient was considered to be immunized against a broad range of HLA-antigens if more than 25% of donors showed a positive result, as described previously[19,21].

Statistical analysis

The mean of the results of every test was classified as either negative or positive. For every combination of 2 tests a 2x2 table was generated and Pearson's chi-squared was calculated to test for independence between two tests. All P-values are two-sided and P-values ≤ 0.05 were considered statistically significant.

Results

ELISA versus LCT

Of 192 serum samples collected before patients received a random platelet transfusion 185 could be analyzed in the ELISA and LCT. The results of the ELISA and LCT were significantly correlated ($\chi^2 = 8.6$; P = 0.003)(Table 2a). Of the 19 serum samples that showed a positive ELISA and a negative LCT a positive LIFT and PIFT could be demonstrated in 12 (63%) and 5 (26%) respectively. Of the 10 serum samples that showed a negative ELISA and a positive LCT a positive LIFT and PIFT could be demonstrated in 1 (10%) and 0 (0%), respectively.

Table 2. The 2 x 2 - tables show the comparison of the results of every combination of two tests, together with Pearson's chi-squared test and the corresponding P-value

a.

Test	LCT (*n*)		
	Negative	**Positive**	**Total**
ELISA			
Negative	150	10	160
Positive	19	6	25
Total	169	16	185

b.

Test	LIFT (*n*)		
	Negative	**Positive**	**Total**
ELISA			
Negative	148	13	161
Positive	7	18	25
Total	155	31	186

c.

Test	PIFT (*n*)		
	Negative	**Positive**	**Total**
ELISA			
Negative	155	6	161
Positive	14	11	25
Total	169	17	186

d.

Test	LIFT (*n*)		
	Negative	**Positive**	**Total**
LCT			
Negative	149	24	173
Positive	9	7	16
Total	158	31	189

e.

Test	PIFT (*n*)		
	Negative	**Positive**	**Total**
LCT			
Negative	162	11	173
Positive	10	6	16
Total	172	17	189

f.

Test	PIFT (*n*)		
	Negative	**Positive**	**Total**
LIFT			
Negative	156	5	161
Positive	19	12	31
Total	175	17	192

a = ELISA vs. LCT: $\chi^2 = 8.6$; $P = 0.003$
b = ELISA vs. LIFT: $\chi^2 = 63.7$; $P < 0.001$
c = ELISA vs. PIFT: $\chi^2 = 42.3$; $P < 0.001$
d = LCT vs. LIFT: $\chi^2 = 9.53$; $P = 0.002$
e = LCT vs. PIFT: $\chi^2 = 17.4$; $P < 0.001$
f = LIFT vs. PIFT: $\chi^2 = 40.8$; $P < 0.001$

ELISA versus LIFT

ELISA and LIFT could be analyzed in 186 of 192 serum samples drawn before a platelet transfusion. The results of the ELISA and LIFT correlated significantly (χ^2 = 63.7; P < 0.001) (Table 2b). Of the 7 serum samples that showed a positive ELISA and a negative LIFT a positive LCT and PIFT could be demonstrated in 0 (0%) and 1 (14%), respectively. Of the 13 serum samples that showed a negative ELISA and a positive LIFT a positive LCT and PIFT could be demonstrated in 1 (7.7%) and 2 (15%) platelet transfusions.

ELISA versus PIFT

ELISA and PIFT were analyzed in 186 of 192 serum samples drawn before a platelet transfusion. The ELISA and PIFT correlated significantly (χ^2 = 42.3; P < 0.001) (Table 2c). Of the 14 serum samples that showed a positive ELISA and a negative PIFT a positive LCT and LIFT could be demonstrated in 0 (0%) and 8 (57%), respectively. Of the 6 serum samples that showed a negative ELISA and a positive PIFT a positive LCT and LIFT could be demonstrated in 0 (0%) and 2 (33%) platelet transfusions.

LCT versus LIFT

LCT was analyzed with LIFT in 189 serum samples drawn before a platelet transfusion. LCT and LIFT showed a significant correlation (χ^2 = 9.53; P = 0.002) (Table 2d). The LCT was positive in 9 (4.7%) samples when LIFT was negative, while the LIFT was positive in 24 (13%) when LCT was negative.

LCT versus PIFT

LCT was analyzed together with PIFT in 189 serum samples drawn before a platelet transfusion.
LCT and PIFT were significantly correlated (χ^2 = 17.4; P < 0.001) (Table 2e). LCT was positive in 10 (5.3%) samples when PIFT was negative and PIFT was positive in 11 (5.8%) when LCT was negative.

LIFT versus PIFT

Finally in 192 serum samples LIFT was analyzed with PIFT. LIFT and PIFT correlated significantly (χ^2 = 40.8; P < 0.001) (Table 2f). A positive LIFT was found in 19 (9.9%) samples when PIFT was negative and a positive PIFT was found in 5 (2.6%) when LIFT was negative.

Discussion

In this study we compared three different techniques that are, with some modifications, commonly used for the detection of alloimmunization during platelet transfusion therapy. These techniques differ in several aspects: sensitivity, specificity, performance time, costs, materials and the necessity of recent donor blood. In the immunofluorescence tests (LIFT and PIFT) and in the LCT antibodies are detected reactive against lymphocytes or platelets, mostly against HLA antigens but not exclusively. For this reason PIFT and MAIPA, that also detect platelet specific

antibodies, are often added to LIFT and LCT. Here we used an immunofluorescence technique in which the detection of antibodies against lymphocytes and platelets are combined. The ELISA (Quikscreen™) only detects HLA class I antibodies, which could be a drawback, if platelet transfusion therapy did not very rarely produce antibodies against human platelet antigens (HPA) without the presence of antibodies against HLA antigens[32, 33].

With respect to the technical aspects, the ELISA (Quikscreen™) has several advantages. The average time to execute this test is 2 to 3 hours; no donor lymphocytes or platelets are needed and the test result is objectively generated by absorbometry, which results in lower costs.

Most reports on this ELISA have been described in organ transplant patients[26 - 28] most of whom do not receive platelet transfusion therapy. Only one report, to the best of our knowledge, describes the characteristics of this test in patients with hematological diseases[30]. These patients, however, were known with refractoriness against platelets, experiencing febrile transfusion reactions or had in their sera nonspecific hemagglutination in an indirect antiglobulin test. Our study was performed prospectively in 192 random serum samples collected in 99 patients with hematological malignancies without selection. We expected to find either weaker antibodies or antibodies with more restricted specificity when sensibilisation occurred. In this way a better insight is generated in the concordance of these tests in a random population of patients with hematological malignancies without increased incidence of alloimmunization by patient selection.

We showed that the ELISA was more frequently positive (19/185) than the LCT showing its superior sensitivity towards detecting alloimmunization, especially since the LIFT was positive in 63% of discordant serum samples. Only in 1 serum sample LCT was positive where ELISA was negative, probably caused by complement mediated cell lysis or by other toxic agents present in patient's serum. The ELISA showed a positive result in 7 samples while the LIFT was negative and the LIFT showed a positive result in 13 samples while the ELISA was negative, more probably pointing to a difference in antigen composition of these 2 tests than by a difference in sensitivity. Comparison of ELISA with PIFT showed a positive ELISA and negative PIFT in 14 samples, while LIFT was positive in 8 (57%). This is probably caused by greater sensitivity for HLA antibodies in ELISA and LIFT than in PIFT, HLA class I antigens being less expressed on platelets than on lymphocytes. In 4 of 6 samples that showed a positive PIFT (2.1% of all serum samples) a negative ELISA, LIFT and LCT was found. This could be caused by antibodies directed against platelet-specific antigens.

In conclusion we demonstrated that the ELISA detects alloantibodies as often as the LIFT and more often than the LCT. Platelet-specific antibodies are not detected in this test, but are infrequent in patients sensitized by random platelet transfusions without the presence of HLA-antibodies[32, 33]. The ELISA, as a screening test, is a faster and cheaper than LIFT, LCT and PIFT without the need for preparation of donor cells and the use of elaborate equipment like a Facscan.

Acknowledgements

We thank Anneke Luijten and Marianne Beije for their help in collecting sera and data. We thank Nicole Bakker for her help on performing the tests and statistical examination of the results. We thank Dew Doekharan for his help with the data management.

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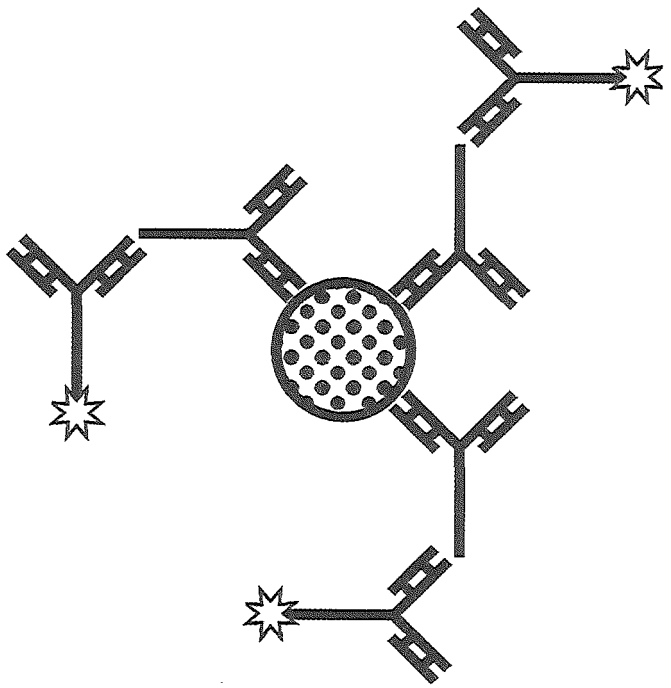
Chapter 4

Platelet-bound immunoglobulins before and after platelet transfusion: measurement of *in vivo* binding



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British Journal of Haematology 1999; 104: 397-402

Abstract

Background

In routine tests to investigate immunological mechanisms as a cause for enhanced destruction of transfused platelets, serum from the patient is tested against a panel of donor lymphocytes and platelets to demonstrate the presence of antibodies against HLA or platelet specific antibodies.

Methods and materials

Here we describe a flow cytometric technique in which in vivo binding of immunoglobulins (Ig) is measured. By comparing the histograms of the platelet suspensions before and after transfusion, four different patterns were obtained: no Ig binding before and after transfusion (pattern 1), pre-existent Ig binding (patterns 2 and 3) or preferential Ig binding to the transfused platelets (pattern 4).

Results

This technique was tested in 164 random platelet transfusions and 34 HLA-matched platelet transfusions. A statistically significant association was found between pattern 1 and a negative result of the indirect platelet immunofluorescence test for the detection of antibodies in the serum taken before the transfusion studied or a good platelet recovery and between pattern 4 and a positive indirect platelet immunofluorescence test or a low platelet recovery.

Introduction

Patients who receive platelet transfusions are at risk of alloimmunization against HLA antigens or platelet-specific antigens[1,2]. These antibodies may interfere with the survival of the transfused platelets[3,4] and may cause febrile transfusion reactions. The presence of alloantibodies against platelets and leucocytes are routinely measured by incubation of the serum of patients with a panel of donor cells (platelets, mononuclear cells and granulocytes). Several methods are used to detect the presence of these alloantibodies. The lymphocyte cytotoxic test (LCT) is based upon the complement-binding capacities of antibodies and subsequent lysis of the panel cells. The enzyme-linked immunosorbent assay (ELISA)[5,6] and the monoclonal antibody-specific immobilization of platelet antigens (MAIPA)[7] technique use enzymes bound to second antibodies and the addition of enzymedependent substrate. In the platelet, lymphocyte and granulocyte immunofluorescence test (PIFT, LIFT and GIFT respectively)[8,9] fluorescein-bound second antibodies are used. Although the sensitivity of these methods may differ, they all are based on the binding of antibodies under *in vitro* circumstances and to panel cells. *In vivo* circumstances, e.g. platelet-bound immune complexes or drug-dependent antibodies which may also jeopardize platelet survival, are ignored in these tests. Testing against panel cells, incubation under *in vitro* circumstances and the inability to detect platelet-bound immunoglobulins not related to alloantibody binding may be technical causes for the discrepancies that are found between the laboratory results and platelet recovery after transfusion in the clinic. Here we describe a technique which measured whether or not immunoglobulins (Ig) to platelets were bound *in vivo* irrespective of the platelet transfusion, as is the case with platelet-bound immune complexes or drug-dependent antibodies, or if binding occurs preferentially to the transfused platelets, as do alloantibodies. Furthermore we studied how these different patterns of Ig binding correlated with the indirect PIFT and with the platelet recovery after transfusion.

Methods and materials

Patients

164 random platelet transfusions in non-refractory patients and 34 HLA-matched platelet transfusions in refractory patients were studied. All patients had haematological malignancies and were thrombocytopenic because of their disease or because of the therapy they received. In all patients a platelet transfusion[9] was given prophylactically if the platelet count was 10 x 10^9/l or less and at hi8gher counts in the

case of clinical bleeding or medical intervention. A random platelet transfusion consisted of five ABO-matched nylon-wool-filtered platelet units and consisted of a mean of 285 x 10^9 platelets per transfusion.
Patients received HLA-matched transfusions if a low platelet recovery after at least two random platelet transfusions was found and anti-HLA antibodies were detectable in the indirect immunofluorescence test against a majority or all of the five panel lymphocytes and platelets. One unit of a HLA-matched platelet transfusion consisted of 289-595 x 10^9 platelets.

Transfusion results

Before and after platelet transfusion the platelet concentration was measured. Of most platelet transfusions the platelet recovery (P.R.) was determined as follows[10,11]:

$$\text{Recovery (\%)} = \frac{\text{Platelet increment } (10^9/\text{l}) \text{ x blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \text{ x } 100$$

A low 1 h recovery was defined as a recovery of <20%. In the few patients of whom the length or body weight was not determined a low recovery was determined as an increment of <15 x 10^9/l.

IVBI-PIFT

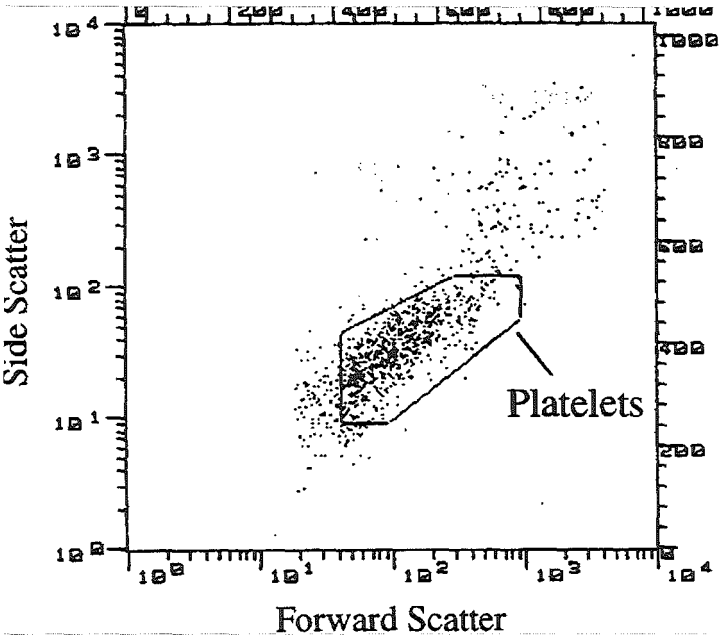
For the detection of *in vivo* binding of immunoglobulins (IVBI) to platelets by the platelet immunoflourescence test (IVBI-PIFT) the results of three suspensions per test were compared. The first platelet suspension was obtained by taking 5 ml of EDTA-anticoagulated blood before the patient received the platelet transfusion (suspension I). The second platelet suspension was obtained by using 1 ml of the leftover donor platelets from the transfusion bag (suspension II). The third platelet suspension was obtained by taking 5 ml of EDTA-anticoagulated blood from the patient 1 h after the platelet transfusion (suspension III). The platelet count was measured in suspension I and III. The three cell suspensions were left overnight at 4^0C.
Platelet-rich plasma (PRP) was obtained by centrifuging the blood containing the platelet suspensions I and III at 400 *g* for 10 min. All centrifugation steps were carried out at 20^0C in a temperature controlled centrifuge (Rotixa RP, Hettich, Germany). The platelet suspensions I, II and III were washed twice in EDTA-PBS-BSA (0.027 M Na_2-EDTA in 0.013 M phosphate-buffered 0.0145 M NaCl, pH 7.4, containing 0.2% bovine serum albumin), resuspended in EDTA-PBS-BSA and adjusted to a concentration of 100 x 10^6/ml. To 50 µl of each platelet suspension 50 µl of a diluted

mixture of fluorescein-conjugated goat anti-IgG (FITC) (NORDIC, Tilburg, The Netherlands) was added, mixed and incubated for 30 min in the dark at room temperature. Then the suspension was washed three times by adding 0.85 ml EDTA-PBS-BSA and by centrifuging at 250 *g* for 7 min. To sort erythrocytes and cell remnants 10 µl LDS-PI (5 µl PI stock, 4 mg propidium iodide in 1 ml PBS (Sigma Company, St. Louis, U.S.A.), together with 5 µl LDS stock, 0.2 mg LDS 751 in 1 ml methanol (Applied Laser Technology, Maarheze, The Netherlands), in 10 ml EDTA-PBS-BSA) was added before the platelet fluorescence was measured by flow cytometry. All tests were performed in duplicate.

Flow cytometry

Five-parameter flow cytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, U.S.A.), which was equipped with an air-cooled argon laser emitting 488 nm at 15 mW. Forward light scatter (FSC) was detected by a solid-state silicon detector, sideways light scatter (SSC) by one photomultiplier tube, FITC by a second photomultiplier tube and a 530 nm bandpass filter, PE by a third photomultiplier tube and a 585 nm bandpass filter (FL2), Laser Dye Solution (LDS-751) by a third photomultiplier tube and a >650 nm bandpass filter (FL3). LDS, which stains DNA and RNA was used to eliminate the erythrocytes from the analysis. From every test-tube 8000 events (during flow cytometry a live-gate is used to select platelets by their FSC, SSC and FL3 characteristics) were collected, of which the data were stored in list mode and analysed using Consort 30 Software (B.D.I.S.).
During the analysis LDS-PI (FL3) was used to diminish background interference of DNA and RNA remnants[12], and the platelets were discriminated from granulocytes and lymphocytes by their FSC and SSC characteristics (Figure 1).

Figure 1. Forward light scatter (horizontal axis) versus sideward light scatter (vertical axis)



Standard live gate set around the platelets.

Of each transfusion a combined histogram was made in 'overlay histograms' of the logarithmical pattern obtained from platelet suspension I, II and III and visually scored.

Indirect PIFT.

To demonstrate HLA and other platelet-reactive antibodies we used a combined platelet and lymphocyte immunofluorescence test (indirect PIFT and LIFT) with flow cytometric analysis, according to Sintnicolaas *et al.*[8] and Gratama *et al.*[13].

Statistical methods.

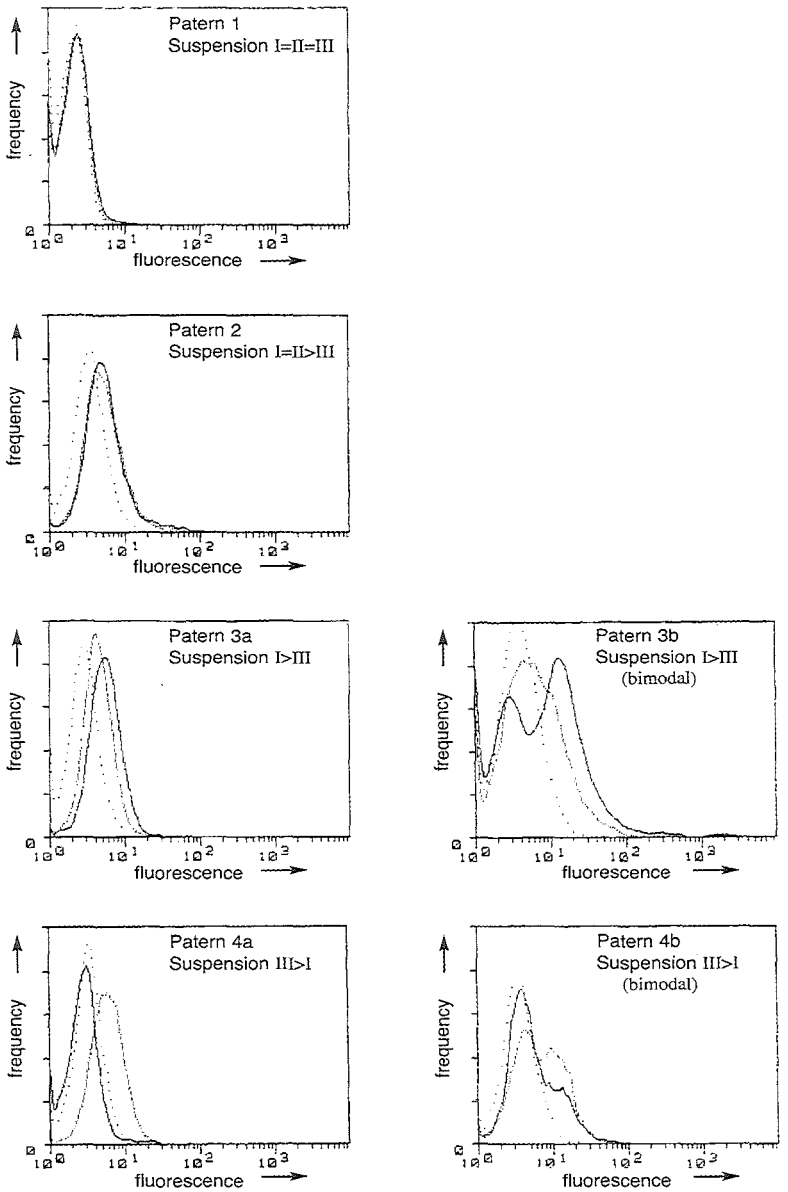
To test the correlation between the results of the IVBI-PIFT and those of the indirect PIFT and between the IVBI-PIFT and the platelet recovery, the Pearson chi-squared test was used.

Results

Interpretation of the patterns

By comparing the results of suspensions I, II and III from each platelet transfusion, four different patterns were seen (Figure 2). Pattern 1 shows an identical histogram of the suspensions before and after the transfusion compared to the histogram of the platelets from the transfusion bag (negative control). Pattern 2 shows a similar shift to the right of both the histograms obtained before and after platelet transfusion compared to the histogram of the platelets from the transfusion bag. Pattern 3 shows a shift to the right of the histogram obtained before transfusion compared to the histogram obtained after transfusion (and the negative control). In a variant of pattern 3 (pattern 3b) a bimodal distribution of the histogram before transfusion is seen. Pattern 4 shows a shift to the right of the histogram obtained after transfusion compared to the histogram obtained before transfusion (and the negative control). Of this pattern also a bimodal distribution of the histogram after transfusion was seen (pattern 4b).

Figure 2. The patterns observed in the IVBI-PIFT



See text for explanation of the patterns.

IVBI-PIFT versus indirect PIFT

We tested 164 random transfusions and compared each of the four different patterns described above with the results of the indirect PIFT from the serum drawn before the transfusion studied (Table I). The indirect PIFT was scored negative if <30% of the panel cells reacted positive, and the indirect PIFT was scored positive if 60% or more of the panel cells reacted positive. Not shown are the transfusions that reacted positive against 30–60% of the panel donors (antibodies with restricted specificity). The serum from patients whose transfusion showed pattern 1 (no Ig binding before or after transfusion) was negative in 92% by the Indirect PIFT. The remaining patterns scored positive as follows: pattern 2 (Ig binding before the transfusion), 65%, for pattern 3 (Ig binding before the transfusion but less binding after transfusion) 84%, and for pattern 4 (preferential binding to the transfused platelets) 63%. The difference between pattern 1 (a negative indirect PIFT) versus pattern 4 (a positive indirect PIFT) was significant (P=0.001). This was also true if pattern 1 and pattern 3 (both indicating no binding of IgG preferentially to the transfused platelets) were taken together (P=0.002).

Table 1. IVBI-PIFT versus indirect PIFT

	Indirect PIFT < 30%	Indirect PIFT > 60%
	n	*n*
Pattern 1	60	5
Pattern 2	19	10
Pattern 3	22	4
Pattern 4	12	7
Total	113	26

Comparison between the four different patterns obtained in the IVBI-PIFT and negative and positive results of the indirect PIFT of the serum drawn within 1 week before the transfusion. Shown are the number of transfusions. In 25 transfusions the indirect PIFT resulted in >30% or <60% of panel cells positive (not shown).

Random platelet transfusions and recovery

We compared the four different patterns obtained from 164 random platelet transfusions with the platelet recovery after 1 h (Table 2). Pattern 1 was seen in 50/72 transfusions (69%) with a recovery >20%. Pattern 2 was seen in 54%; pattern 3, 89%; and pattern 4, 33%. The difference in the distribution of pattern 1 and pattern 4 with a recovery of more or less than 20% respectively was significant (P=0.002), and this was also the case if pattern 1 and pattern 3 were taken together (P=0.0002).

Table 2. IVBI-PIFT versus recovery

	Recovery > 20%	Recovery ≤ 20%
	n	*n*
Pattern 1	50	22
Pattern 2	23	19
Pattern 3	23	3
Pattern 4	8	16
Total	104	40

Comparison between the four different patterns obtained in the IVBI-PIFT and the platelet recovery after 1 h. Shown are the number of transfusions.

HLA-matched platelet transfusions in alloimmunized patients

Table 3 shows the results of 34 HLA-matched platelet transfusions in immunized patients. All of these HLAmatched transfusions had a recovery > 20% or the platelet concentration increased to > 15 x 10^9/l platelets. The degree of HLA matching with regard to the class 1 antigens (A and B locus) was scored according to Duquesnoy *et al*[14]: identical: all four antigens in the donor were identical to the recipient; compatible: three or less antigens were detected in the donor, all present in the recipient; crossreacting: antigens in the donor if not identical, cross-reactive with the recipient.

Of 29 HLA-matched transfusions the ABO blood group of the platelet donor was known and ABO incompatibility was present in seven transfusions. None of these transfusions had pattern 1, one had pattern 2, one had pattern 3 and five had pattern 4.

Table 3. HLA-matched transfusions in alloimmunized patients

	Identical	Compatible	Crossreacting	*Total*
Pattern 1	1	6	2	9
Pattern 2	2	8	1	11
Pattern 3	1	3	1	5
Pattern 4	0	3	6	9
Total	4	20	10	34

Frequency of the four different patterns obtained in the IVBI-PIFT of 34 HLA-matched platelet transfusions, classified by the degree of HLA class I match.

Discussion

The use of platelet transfusions continues to increase[15,16], but the reason for enhanced destruction of transfused platelets often remains difficult to determine. Alloimmunization is one of the factors that can cause shortened survival of transfused platelets. Several tests are used to detect alloimmunization. The LCT detects antibodies against HLA epitopes[17]. Platelet-specific antibodies can be measured in the PIFT[8], MAIPA[7] or ELISA[5,6]. All these tests use panel cells. The correlation of the results of these tests with the platelet recovery is poor. Several reasons for this phenomenon can be noted: in these tests panel cells are used and not the transfused platelets, binding under *in vitro* circumstances is measured, platelet-bound immune complexes may be missed, drug-induced antibodies, which may also unfavourably affect platelet survival, are not measured, and the sensitivity of the different tests (mostly not quantified) may not be predictive for the rapidity of destruction of the platelets, as not only the amount of antibody but also the (sub)class may play a role. And last, but not least, non-immunological factors for shortened survival of platelets, such as bleeding and hepatosplenomegaly cannot always be recognized and eliminated in a clinical study. Here we describe a technique which has theoretical and practical advantages compared to the methods mentioned above. Firstly, Ig binding to the transfused platelets under *in vivo* circumstances is measured. Secondly, comparison of the Ig binding before and after transfusion discriminates between pre-existent higher Ig binding, e.g. by immunecomplexes, autoantibodies or drug-induced antibodies on one hand (patterns 2 and 3) and preferential binding to the transfused platelets (pattern 4) as can be seen if alloantibodies are present on the other hand. Practical advantages compared to the standard methods are that the test can be performed within 4 h, no panel platelets are needed, and no extra amount of blood has to be drawn from the patient. Interpretation of the results is based on the comparison of the flow cytometric patterns obtained from the PIFT before and after transfusion using the pattern of the PIFT of the platelets from the transfusion bag as a negative control. In this series four different patterns were observed, a fifth pattern (shift to the right of the histogram of the platelets from the transfusion bag compared to the histograms of preand post-transfusion platelets) was not seen and would make the test uninterpretable. We have chosen in this study for a visual scoring, because bimodal distributions were sometimes seen in patterns 3 and 4, which made validation of a quantitative approach more difficult and only possible in a larger study. For the evaluation of the IVBI-PIFT we compared the different patterns to the results of the indirect PIFT and the recovery in random transfusions. The comparison of the IVBI-PIFT and the indirect PIFT showed a correlation between pattern 1 and 3 (no preferential binding to the transfused

platelets) and a negative indirect PIFT on one hand and pattern 4 (binding to the transfused platelets) and a positive indirect PIFT on the other. In pattern 2 (pre-existent Ig binding) the correlation is less clear. In these cases binding of alloantibodies cannot be excluded as there was already a shift to the right. Discrepancies between the IVBI-PIFT and the indirect PIFT can be explained by a difference in substrate (transfused platelets versus panel platelets), a difference in environment (*in vivo* and *in vitro*), and a difference in the reading of the tests (visual or quantitative). The recoveries of the transfusions with patterns 1 and 3 were significantly better than those of the transfusions with pattern 4, which is to be expected, since pattern 4 is explained by binding of alloantibodies to the transfused platelets. However, pattern 1 was also seen with a recovery <20%, pointing to destruction or loss by other than immunological factors. An other explanation is that pattern 1 also resulted from a destruction of the transfused platelets within 1 h of transfusion (recovery 0%). However, pattern 4 was observed while the platelet transfusion resulted in a recovery > 20%. This implicated, and was also deduced from the *in vitro* tests, that *in vivo* binding of antibodies to transfused platelets does not necessarily lead to enhanced destruction. Differences in the amount of bound IgG or subclass make-up are explanations for this phenomenon. Pre-existent IgG binding to platelets may be compatible with a normal 1 h survival, but a shortened survival over a longer period cannot be excluded. All four patterns were encountered in the HLA-matched transfusions, all of which had a recovery of >20%. Pattern 4 may be caused by the presence of crossreacting HLA antibodies, platelet-specific antibodies or ABO antagonism. In conclusion, the technique described here detects whether *in vivo* Ig was bound to the platelets before transfusion and if possible changes occurred after the platelet transfusion. If the test is used as a screening test to evaluate the cause of refractoriness, it may discriminate between alloimmunization and other immunological mechanisms that can interfere with platelet survival.

Acknowledgements

We thank Ferdinand de Winter for his data collection and analysis, and Patricia van den Broek and Petra van der Spoel for their technical assistance.

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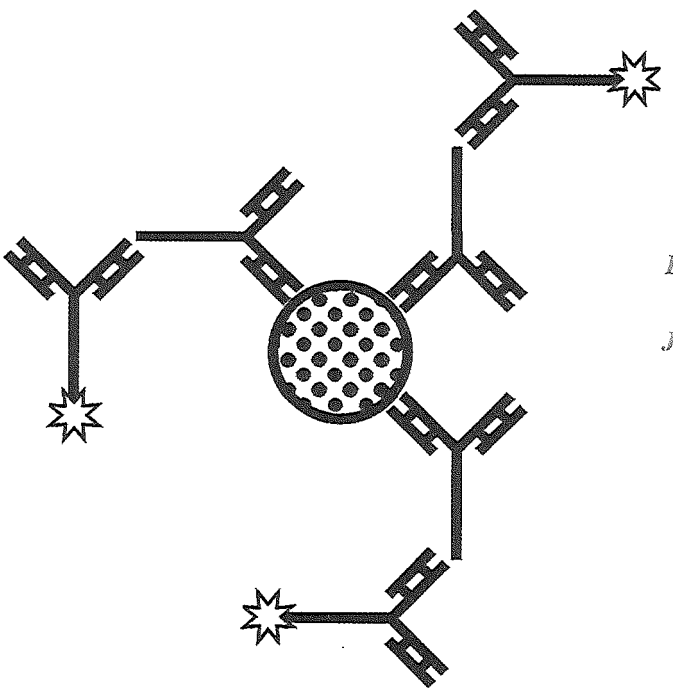
Chapter 5

Visual scoring versus histogram subtraction of *in vivo* binding of immunoglobulins against platelets after transfusion



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Clinical Cytometry 2004; 57B: 40-46

Abstract

Background

We described a technique, in vivo binding of immunoglobulins in the platelet immunofluorescence test (IVBI-PIFT), that detects IgG bound in vivo to transfused platelets. The visually scored results of this technique, however, are susceptible to interobserver variation. Here we describe a more objective method to generate results in the IVBI-PIFT.

Methods and materials

We studied 201 samples in 120 patients with hematological malignancies in the IVBI-PIFT. Histogram subtraction, fluorescence (anti-IgG-FITC) histogram before platelet transfusion subtracted from the histogram after platelet transfusion, was compared to visual scoring (pattern 1: no enhanced fluorescence before and after transfusion; pattern 2: enhanced fluorescence both before and after platelet transfusion; pattern 3: enhanced fluorescence before transfusion; pattern 4: enhanced fluorescence after transfusion, interpreted as alloimmunization). Following histogram subtraction, the number of remaining events (EPS) and the mean amount of fluorescence of these remaining events (MCPS) were used and compared with the visual scoring and with platelet survival after transfusion.

Results

In 26 (13%) of the 201 samples studied in the IVBI-PIFT less than 3 of 5 observers agreed on the visually scored pattern. In the 175 (87%) remaining samples histogram subtraction showed a significant differentation between pattern 4 and patterns 1 and 2 by using EPS, while patterns 4 and 3 were distinguished by using the MCPS. The combination of EPS and MCPS differentiated best between pattern 4 and patterns 1, 2 and 3 (sensitivity 73%, specificity 96%, positive predictive value 79% and negative predictive value 95%). Predictive value for platelet recovery after 1 and 16 hours of both pattern 4 from the visual scoring method and the results of histogram subtraction, however, was poor.

Conclusion

We describe an objective method of histogram subtraction that correlates well with the visual scoring method of the IVBI-PIFT.

Introduction

Patients who receive platelet transfusions are at risk of alloimmunization against HLA antigens or platelet-specific antigen[1,2]. These antibodies may interfere with the survival of the transfused platelets[3,4] and may cause febrile transfusion reactions. The presence of alloantibodies against platelet and leucocyte antigens are routinely measured by incubation of the serum of patients with a panel of donor cells (platelets, mononuclear cells and granulocytes). Several methods have been described to detect the presence of these alloantibodies: the Lymphocytotoxic Test (LCT)[5-7], microscopic Platelet Suspension Immunofluorescence Test (PSIFT)[8], Enzyme-Linked Immunosorbent Assay (ELISA)[9–12], Monoclonal Antibody-specific Immobilization of Platelet antigens Assay (MAIPA)[13-15], monoclonal ^{125}I-labeled anti-IgG assay (^{125}I-MA)[16], flow cytometric Platelet and Lymphocyte Immunofluorescence Test (PIFT respectively LIFT)[17–21], ^{51}Cr platelet lysis assay[22] and Platelet Radioactive Antiglobulin Test (PRAT)[23]. Although the sensitivity of these methods may differ, they all are based on the binding of antibodies under *in vitro* circumstances and to panel cells. *In vivo* circumstances, e.g. platelet-bound immune complexes or drug-dependent antibodies which may also adversely affect platelet survival, are not evaluated by these tests. Testing against panel cells rather than against platelets of the transfused donor(s), incubation under *in vitro* circumstances and the inability to detect platelet-bound immunoglobulins (Ig) not related to alloantibody binding may be technical causes for the discrepancies that are found between the laboratory results and platelet recovery after transfusion in the clinic. Previously we have described a test that detects IgG bound to (transfused) platelets *in vivo*, the In Vivo Binding of Immunoglobulins in the Platelet Immunofluorescence Test (IVBI-PIFT)[24]. In this work the results of the IVBI-PIFT were visually scored by a single blinded observer. We demonstrated that preferential binding of immunoglobulins after platelet transfusion (pattern 4) was associated with significantly lower platelet recoveries than when no binding of immunoglobulins after platelet transfusion took place (pattern 1). The introduction of the IVBI-PIFT in our clinic revealed a considerable inter-observer variability for the visual scoring of of the 4 different patterns. Here we evaluate the use of histogram subtraction as an objective alternative for the subjective visual comparison of paired histograms, and correlated the results of both methods of data interpretation with the corresponding platelet recoveries at 1 and 16 hours after transfusion.

Methods and materials

Samples

From October 1994 until August 2000 201 paired serum samples (before and after transfusion) were studied in 120 random patients, mean 1.7 transfusions per patient (range 1-7), who were treated for a malignant lymphoma or leukemia in our hospital. Patients received a platelet transfusion of random donors prophylactically (i) when platelet count was less than 10 x 10^9/l, (ii) before an intervention or (iii) because of clinical manifest bleeding at higher platelet counts. The serum samples were collected before and one hour after the platelet transfusion.

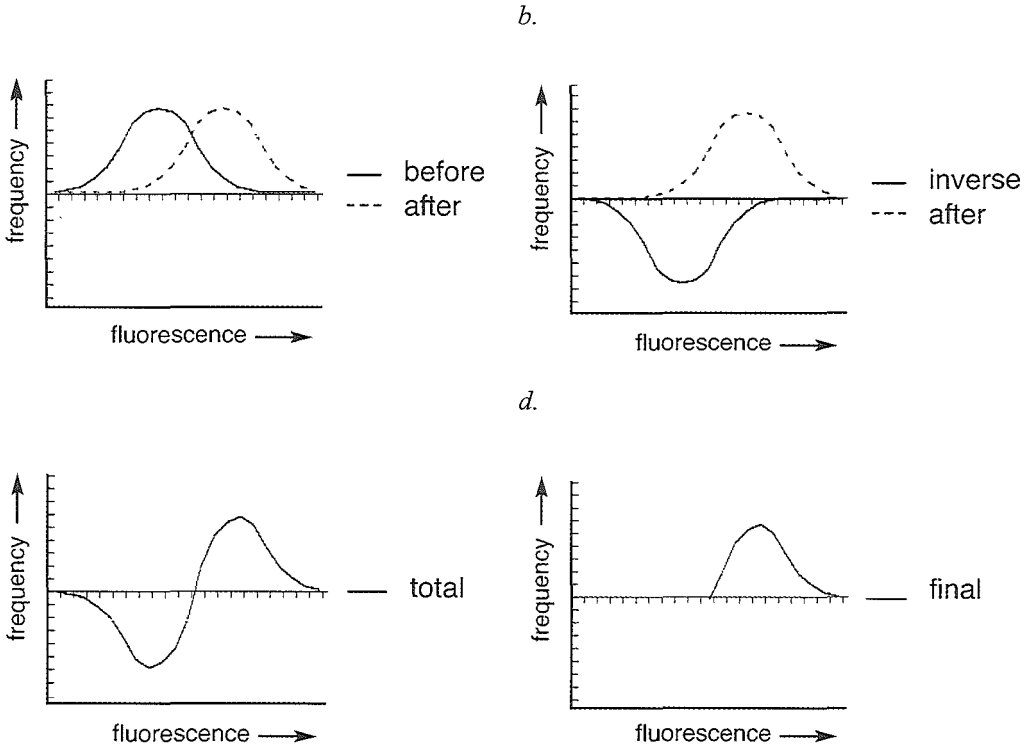
IVBI-PIFT

The IVBI-PIFT method has been described elswhere[24]. In short: after the collection of whole blood, before and after platelet transfusion, the results of three platelet suspensions after incubation with a mixture of fluorescein-conjugated goat anti-IgG (FITC) were used: I: before transfusion, II: the transfusion bag and III: 1 hour after transfusion. Per suspension a histogram, showing the distribution of IgG-fluorescence on a logarithmic scale, was obtained by collecting 4-parameter flow cytometry data from 8,000 platelets. Of each transfusion an overlay histogram was composed from the fluorescence of platelet suspensions I, II and III. All tests were performed in duplicate.

Histogram subtraction

The histograms of the IgG-fluorescence of suspension I (prior to transfusion) was subtracted from the histogram of suspension III (post transfusion). This was done by subtracting the number of platelets for each fluorescence channel separately (linear scale of 256 histogram channels). If differences between the corresponding channels of histogram I and III resulted in negative values these were replaced by zero events in the subtraction histogram (Figure 1).

Figure 1. Stepwise deduction of the histogram before transfusion from the histogram after transfusion



a. Both curves in one figure
b. Curve before transfusion is inverted with respect to the x-axis
c. Addition of the number of events per fluorescence range
d. The negative part of the curve is removed

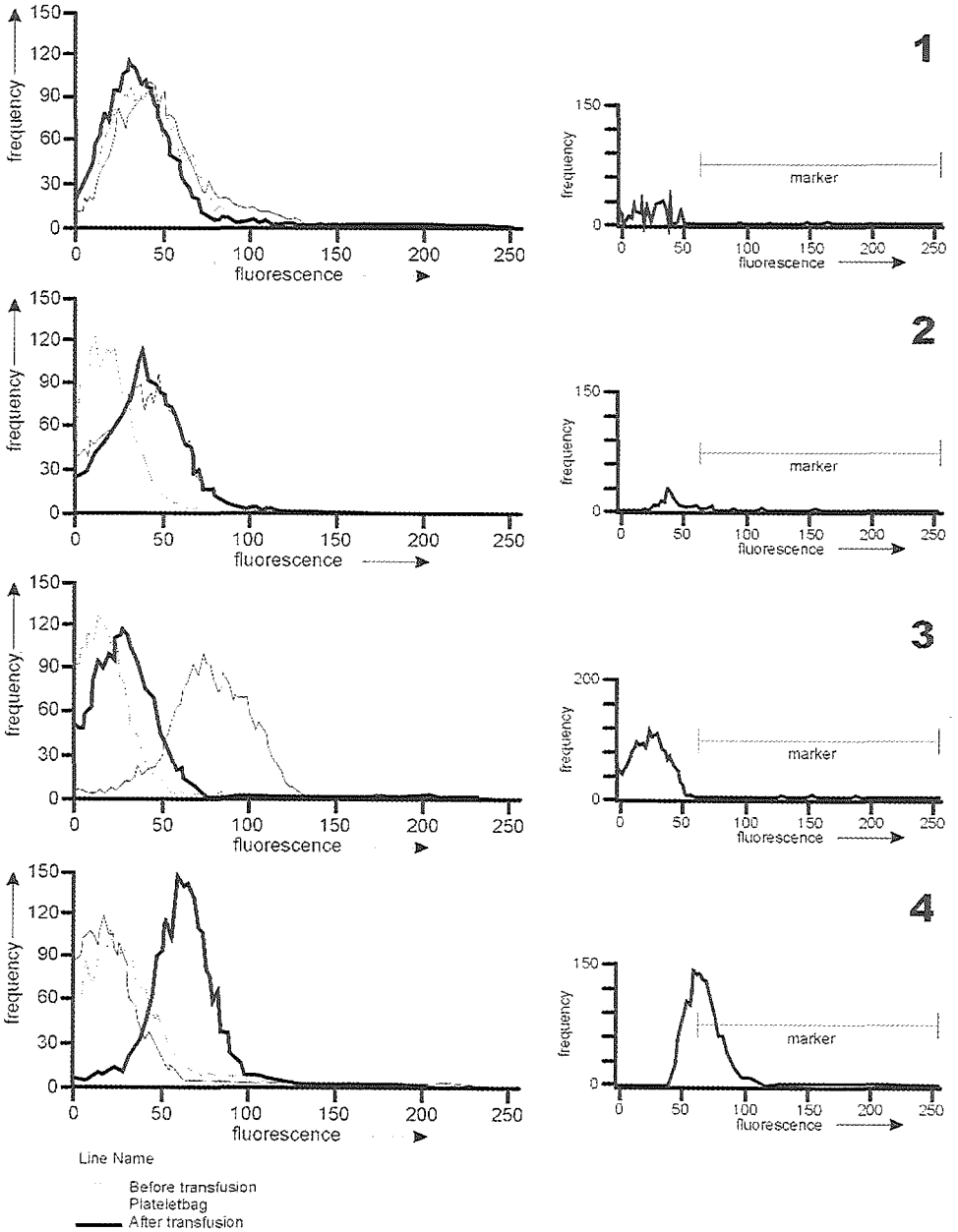
Histogram II was used as a negative control (see below). This leaves a subtraction histogram that can be characterised by the number of remaining events (= total number of platelets of all fluorescence channels after this subtraction) and the mean fluorescence of the remaining events. First, "*Events post subtraction*" (EPS) was defined as the average of all remaining events of both (duplicate) subtraction histograms. Second, "*Mean channel post subtraction*" (MCPS) was defined as the average of the mean channel values of both (duplicate) subtraction histograms.

Visual scoring (Figure 2)

Patterns were scored independently and blinded by 5 observers according to the following definitions:

Pattern 1: similar shapes of histograms of the suspensions I, II and III (interpretation: no alloimmunization). Pattern 2: strongly resembling shifts to the right of both the histograms of suspension I and III relative to suspension II (interpretation: binding of IgG not related to alloimmunization). Pattern 3: a shift to the right of the histogram of suspension I relative to suspension III (interpretation: preferential binding of IgG to own or previously transfused platelets). Pattern 4: a shift to the right of the histogram of suspension III relative to suspension I (interpretation: alloimmunization) (Figure 2)[24]. When 3 or more of all 5 observers indicated the same pattern for both (duplicate) overlay histograms per platelet transfusion, this was considered as the consensus pattern and was used for further analysis.

Figure 2. Visual scoring and histogram subtraction of the IVBI-PIFT



The 4 patterns observed in the IVBI-PIFT by visual scoring (on the left) and the results of the of histogram subtraction, after platelet transfusion minus before platelet transfusion (on the right).

Platelet transfusion results

A successful platelet transfusion was defined as a recovery of platelets after transfusion of 20% or more after 1 hour or 10% or more after 16 hours[25,26]:

$$\% \text{ recovery} = \frac{\text{Platelet increment } (10^9/l) \text{ x blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \text{ x } 100$$

Statistical methods.

EPS and MCPS as continous variables were compared with the consensus patterns 1, 2, 3, and 4 using the Kruskal-Wallis test. Pearson's chi-squared test or Fisher's exact test, whichever appropriate, was used to compare the EPS and MCPS, after dichotomization into the lower two-thirds and the higher one-third, with the 4 consensus patterns. Univariate logistic regression was used to calculate P-values for differences in recovery for the visual and histogram subtraction method with adjustment for multiple transfusions in single patients. All P-values are two-sided and P-values ≤ 0.05 were considered statistically significant.

Results.

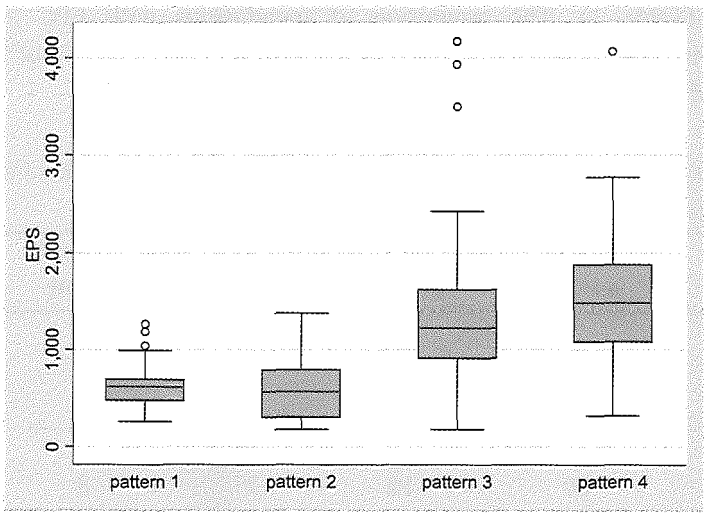
Visual scoring vs. EPS and MCPS results

In 201 transfusions the results of the IVBI-PIFT were both visually scored and generated by histogram subtraction. In 26 (13%) of the transfusions studied in the IVBI-PIFT less than 3 of 5 observers agreed on the pattern so that no consensus was reached. The comparison of the histogram subtraction results with the visually scored patterns of the IVBI-PIFT was, therefore, performed in 175 transfusions administered to 111 patients.

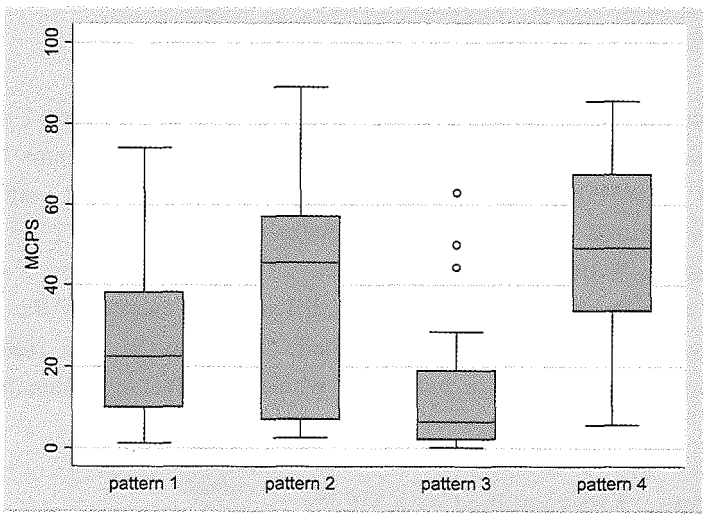
Expectedly, the median number of EPS was significantly higher for transfusions showing pattern 4 (alloimmunization) as compared to those with pattern 1 and 2 (no alloimmunization) 1480, 615 and 566, respectively (Figure 3A), but no difference was found with transfusions showing pattern 3, of which the median EPS was 1222. The median value of MCPS was significantly higher in pattern 4 than in pattern 3, 49 and 7 respectively (Figure 3B), but no difference was found with transfusions showing pattern 1 and 2, MCPS being 23 and 46, respectively.

Figure 3. **Box and whisker plot of histogram subtraction vs. visual scoring method.**

a.



b.



The line in the middle of the box represents the median of the data. The box extends from the 25th percentile ($x_{[25]}$) to the 75th percentile($x_{[75]}$), the so-called interquartile range (IQR). The lines emerging from the box are called the whiskers and they extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point $\leq x_{[75]} + 1.5 \times$ IQR. The lower adjacent value is defined as the smallest data point $\geq x_{[25]} - 1.5 \times$ IQR. Observed points more extreme than the adjacent values, if any, are individually plotted.

a. EPS vs. pattern 1, 2, 3 and 4

b. MCPS vs. pattern 1, 2, 3 and 4

Dichotomization of the transfusions into a upper one-third (N = 58) with EPS ≥ 1040 and the lower two-third (N = 117) with EPS < 1040 revealed the following correlations (Table 1). The proportion of transfusions with EPS ≥ 1040 was significantly higher for pattern 3 and 4 than for pattern 1 and 2 (i.e., 52 of 72 [72%] *vs.* 6 of 103 [6%] transfusions, respectively, P < 0.0001).

Table 1. EPS and MCPS (after dichotomization into a lower two-third and a higher one-third group) vs. patterns 1, 2, 3 and 4

	Pattern 1 *n* = 82	**Pattern 2** *n* = 21	**Pattern 3** *n* = 42	**Pattern 4** *n* = 30	*Total* *n* = 175
EPS†					
< 1040	79	18	14	6	117
≥ 1040	3	3	28	24	58
MCPS‡					
< 37	60	10	39	8	117
≥ 37	22	11	3	22	58

† *P < 0.0001 for pattern 3 and 4 vs. pattern 1 and 2*

‡ *P < 0.0001 for pattern 3 vs. pattern 4*

The difference between pattern 3 and 4 on the one hand and patterns 1 and 2 on the other, is caused by the fact that the positions of the data in histograms I and III are divergent for patterns 3 and 4, and to a large extent overlapping for patterns 1 and 2 (Figure 2). In contrast, the proportion of transfusions with MCPS ≥ 37 was clearly higher in pattern 4 than for pattern 3 (i.e., 22 of 30 [73%] *vs.* 3 of 42 [7%] transfusions, respectively, P < 0.0001). This difference is caused by the fact that the posstransfusional histogram (III) is situated far more to the right than the pretransfusional histogram (I) in pattern 4, whilst the reverse is true for pattern 3 (Figure 2).

Visual scoring vs. EPS combined with MCPS

A combination of EPS and MCPS was used to generate more distinction between a positive or negative test result. The proportion of transfusions that demonstrated EPS ≥ 1040 and MCPS ≥ 37, these values were described above after dichotomization, was significantly higher for pattern 4 (63%) than for pattern 1 (2%), 2 (14%), and 3 (5%) (P < 0.001) (Table 2). This resulted in a sensitivity of 63%, a specificity of 95%, a positive predictive value of 73% and a negative predictive value of 93% in differentiating pattern 4 from pattern 1, 2 and 3.

Table 2. Combination of EPS with MCPS vs. pattern 1, 2, 3 and 4

	Pattern 1 $n = 82$	**Pattern 2** $n = 21$	**Pattern 3** $n = 42$	**Pattern 4** $n = 30$	***Total*** $n = 175$
EPS ≥ 1040 AND MCPS ≥ 37[†]					
< 1040	80	18	40	11	149
≥ 1040	2	3	2	19	26
MCPS ≥ 1100 AND MCPS ≥ 30[‡]					
< 37	80	19	40	8	147
≥ 37	2	2	2	22	28

Two combinations of cut-off points for EPS and MCPS compared to patterns 1, 2, 3 and 4. Yes = number of transfusions that show EPS ≥ 1040 (1100) AND MCPS ≥ 37 (30); No = all other transfusions.

[†] *P < 0.001 for EPS ≥ 1040 AND MCPS ≥ 37*

[‡] *P < 0.001 for EPS ≥ 1100 AND MCPS ≥ 30*

The combination of EPS and MCPS was investigated further in order to find a cut-off point that showed the best combination of sensitivity, specificity, positive predictive value and negative predictive value. The best cut-off point was found in the combination of EPS ≥ 1100 and MCPS ≥ 30, resulting in a sensitivity of 73%, a specificity of 96%, a positive predictive value of 79% and a negative predictive value of 95%, for differentiating pattern 4 from patterns 1, 2 and 3 (Table 2).

Visual scoring and histogram subtraction vs. platelet recovery (Table 3)

The visual scoring method demonstrated no larger proportion of poor platelet recoveries after 1 and 16 hours for pattern 4 compared to the recoveries of pattern 1, 2 and 3 (P = 0.89 and P = 0.95, respectively). The proportion of poor platelet recoveries after 1 and 16 hours of all patterns differed significantly (P = 0.02) and borderline significantly (P = 0.06), respectively. This was mainly caused by the correlation of pattern 2 with poor platelet recovery after both 1 and 16 hours.

Table 3. Visual scoring of patterns and histogram subtraction (EPS ≥ 1100 AND MCPS ≥ 30) vs. the number of successful transfusions after 1 and 16 hours

	Recovery after 1 hour† n (%)		Recovery after 16 hours‡* n (%)	
	≥ 20%	< 20%	≥ 10%	< 10%
Visual scoring				
Pattern 1	42 (51%)	40 (49%)	50 (64%)	28 (36%)
Pattern 2	2 (11%)	19 (89%)	7 (35%)	13 (65%)
Pattern 3	21 (50%)	21 (50%)	27 (75%)	9 (25%)
Pattern 4	13 (43%)	17 (57%)	18 (62%)	11 (38%)
Histogram subtraction results (EPS ≥ 1100 AND MCPS ≥ 30)				
No	66 (45%)	81 (55%)	84 (61%)	53 (39%)
Yes	12 (43%)	16 (57%)	18 (69%)	8 (31%)

† *Recovery after 1 hour: P = 0.02 for all patterns, P = 0.89 for pattern 4 compared with the other patterns; P = 0.85 for histogram subtraction*

‡ *Recovery after 16 hours: P = 0.06 for all patterns, P = 0.95 for pattern 4 compared with the other patterns; P = 0.42 for histogram subtraction*

* *After 16 hours a total of 12 recovery results were missing: 4 for pattern 1, one for pattern 2, 6 for pattern 3 and one for pattern 4; 10 for negative histogram subtraction and 2 for positive histogram subtraction*

Subsequently, a positive result of the histogram subtraction (EPS ≥ 1100 and MCPS ≥ 30) did not demonstrate an association with poor platelet recovery after 1 and 16 hours (P = 0.85 and P = 0.42, respectively).

Discussion

Of all platelet transfusion 24 to 44% do not result in the expected platelet increment[3,27]. Because platelet transfusion failure is often multicausal in patients with hematological diseases a test that reliably detects alloimmunization can be a step forward in selecting patients in need of HLA-matched or cross-matched platelet transfusions. Recently we have shown that pattern 4 of the IVBI-PIFT significantly correlates with a low recovery of a subsequent platelet transfusion in patients at risk for alloimmunization[24]. The results of the IVBI-PIFT, however, were visually scored, a method subjected to inter-observer variation.

In this study the inter-observer variation of the visually scoring of the patterns, defined as less than 3 of 5 observers reporting the same pattern, was 13% (26 serum samples),

which was in accordance to our experience during clinical practice. In order to study the association of the histogram subtraction with the visual scoring method we compared the results of histogram subtraction with the "golden standard" visual scoring method, in which 3 or more of the 5 observers agreed on the visually scored pattern, and excluded these 26 transfusions from further study. We can not exclude that this exclusion may be selective.

In order to objectivate the results of the IVBI-PIFT histogram subtraction was introduced by subtracting the fluorescence histogram of a serum sample before transfusion from the fluorescence histogram after the platelet transfusion. We previously have studied the effect of subtracting the mean or median of the histograms. This did not lead to a reliable substitution of the visual scoring method (data not published), because of biphasic curves before or after transfusion or because of a substantial difference in the range of the channel numbers with events before or after transfusion. Because of these difficulties we studied the subtraction of the number of events per channel number in 175 samples in which also consensus could be reached by the visual scoring method. This histogram subtraction resulted in the variables EPS and MCPS. The EPS $\geq$ 1040 clearly demonstrated a significant differentiation between pattern 3 and 4 on one hand and 1 and 2 on the other hand. Subsequently pattern 3 and 4 could be differentiated by MCPS $\geq$ 37.

We set out to generate a positive or negative test result of the IVBI-PIFT by combining the EPS and MCPS. When the combination of the dichotomization cut-off for EPS and MCPS was used (EPS $\geq$ 1040 and MCPS $\geq$ 37) a good differentiation between pattern 4 and patterns 1, 2 and 3 could be achieved. Other cut-off points were also tested, which resulted in a best cut-off point characterised by EPS $\geq$ 1100 and MCPS $\geq$ 30. This cut-off point showed a better sensitivity, positive predictive value and negative predictive value than the original cut-off point while maintaining the same specificity. Still 8 (27%) transfusions demonstrating pattern 4 were negative in the histogram subtraction method, and 6 (4%) transfusions demonstrating pattern 1, 2 or 3 were positive in the histogram subtraction method. This cut-off point has no universal meaning because it is sensitive to laboratory dependent factors such as temperature, incubation time, laser emitting wave length and fluorescence antibody concentration, so it should be validated for each laboratory.

Finally, in order to validate the technique the results of the visual scoring method and the histogram subtraction method were compared to platelet recovery after 1 and 16 hours of the studied platelet transfusion. No significant correlation could be demonstrated between pattern 4 of the visual scoring method and recovery after 1 and 16 hours. Pattern 2, however, did demonstrate a significant negative correlation with platelet recovery both after 1 and 16 hours, but the numbers are small. This may indicate the importance of

immune complexes as a causative agent in enhanced destruction of transfused platelets. Pattern 4, defined as increased IgG present on transfused platelets, was interpreted as compatible with alloimmunization and may be used to select patients for HLA-matched platelet transfusions. Here we demonstrate, however, that binding of IgG to transfused platelets does not necessarily lead to enhanced donor platelet destruction. The majority of discrepancies between test results and platelet recovery are probably caused by non-immunological factors causing enhanced platelet destruction present at the time of transfusion[28].

In conclusion we demonstrated that histogram subtraction of the IVBI-PIFT by combining EPS and MCPS is a reliable and objective substitution for visually scored consensus patterns. In this study, however, no correlation was demonstrated between pattern 4 of the visual scoring method or a positive histogram subtraction result and platelet recovery after 1 and 16 hours.

Acknowledgements

We thank Karola van Rooyen for her help with the layout of the figures.

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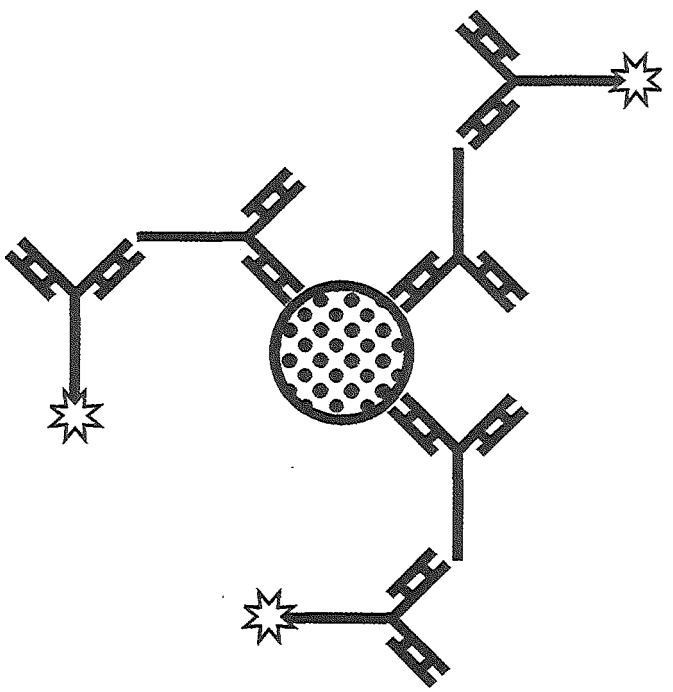
Chapter 6

Immune and non-immune causes of low recovery from leukodepleted platelet transfusions: a prospective study



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Annals of Hematology 2003; 82: 357-362

Abstract

Background

Allo-antibodies against HLA-antigens can be reduced by applying leucodepletion to transfusions. Because the importance of immunological and non-immunological causes of poor platelet transfusion results using leucodepleted transfusions is not clear we conducted a prospective study in an unselected patients population receiving leucodepleted transfusions.

Methods and materials

In 97 patients with hematological malignancies 181 random leucodepleted platelet transfusions were studied for immunological causes of poor platelet transfusion results by calculating the odds ratio of 4 different screening tests for a low platelet recovery. Non-immune causes were also studied by calculating the odds ratio of the most prevalent non-immune causes for a low platelet recovery.

Results

No single screening test showed an association with recovery after 1 and 16 hours following a platelet transfusion. The combination of a positive ELISA and PIFT or a combination of a positive LIFT and PIFT, demonstrating an association with a low platelet recovery after 16 hours, was present in 2% of all platelet transfusions. Of non-immune causes splenomegaly and storage time of platelets of more than 3 days were associated with low platelet recovery after 1 hour and 16 hours being present in 29% and 47% of all platelet transfusions, respectively.

Conclusions

Immunological causes account for a small proportion of poor platelet transfusion results compared to non-immunological causes in a non-selected patient population receiving leucodepleted transfusions.

Introduction

Patients with hematological malignancies may have prolonged periods of thrombocytopenia caused by bone marrow involvement or treatment. Platelet transfusions, mostly from random donors, are given beneath a platelet count of 10 x 10^9/l to reduce the risk of bleeding[1-3]. Many platelet transfusions, however, do not result in the expected platelet recovery because of immunological factors in 18-70% (anti-HLA antibodies, anti-HPA antibodies; ABO-mismatch, circulating immune complexes)[4–19]and non-immunological factors in 33-67% (splenomegaly, bone marrow transplantation, Amphotericin B, Ciprofloxacin, Tacrolimus, Cyclosporine, Vancomycin, Fluconazol, hyperbilirubinemia; total body irradiation; disseminated intravascular coagulation, advanced disease status, fever, sepsis, etc.)[9,11,13–17,19-21]. Sensitization by random donor platelet transfusions against HLA and platelet specific antigens is one of the causes that can be measured and circumvented by the use of platelets compatible for the antigens involved. Leucodepleted transfusions have shown to diminish the prevalence of sensitization against HLA antigens, the most frequent immunological cause of platelet refractoriness[14,22–25]. Several tests are used to demonstrate the presence of allo-antibodies against platelet antigens but the prevalence of positive test results in an unselected patient population receiving leucodepleted transfusions is not clear[6,7,26-34]. Also non-immunological factors that can diminish platelet recovery in an unselected patient population receiving leucodepleted platelet transfusions are not well-defined. In order to clarify these issues we conducted a prospective study in which both immunological causes, as measured by four different tests, and the most prevalent non-immunological causes were related to platelet recovery.

Methods and Materials

Patients

From December 1998 until August 2000 181 serum samples collected from randomly selected platelet transfusions from 97 random patients were tested, mean 1.9 transfusions per patient (range 1-8) who were treated for a malignant lymphoma or leukemia in our referral hospital (Table 1).The serum samples were collected before patients received a random donor platelet transfusion when platelet count was less than 10 x 10^9/l to prevent bleeding, or at higher counts before an intervention or because of clinical manifest bleeding. The median platelet count before transfusion was 11 x 10^9/l (range 4-60 x 10^9/l).

Table 1. Patient characteristics

Characteristics	Category	Number
Sex	Male	61
	Female	36
Age in years	Median	46
	Range	16-76
Diagnosis	ALL	4
	AML	43
	CLL	1
	CML	9
	MM	6
	NHL	18
	HD	4
	MDS	9
	Other	3
Therapy	Chemotherapy	59
	Auto BMT/SCT	10
	Allo BMT/SCT	23
	Other	5

ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphoblastic leukemia; CML = chronic myeloid leukemia; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; HD = Hodgkin's disease; MDS = myelodysplastic syndromes; Auto BMT/SCT = autologous bone marrow transplantation or peripheral stem cell transplantation; Allo BMT/SCT = allogeneic bone marrow transplantation or peripheral stem cell transplantation

Platelet concentrates

Platelet concentrates were obtained from the Sanquin Blood Bank South West Region and were prepared from the blood of 5 HLA-unmatched ABO-matched volunteer donors. The average content of the platelet concentrates was 4 x 10^{11} platelets in 250 ml. The platelet concentrates were filtered by a PLX-5 Asahi filter (Baxter, La Chatre, France) to deplete the transfusion from leucocytes. The average leukocyte number was 0.05 x 10^6 per platelet concentrate (range 0.00-0.20 x 10^6). In patients receiving a bone marrow or peripheral stem cell transplantation the platelet concentrates were irradiated by 25 Gy in order to prevent graft versus host disease (GvHD) by transfused lymphocytes[35]. The platelet concentrates were stored at 22 ^{0}C until used for transfusion. Also erythrocyte concentrates were always leucocyte depleted and irradiated for the same reasons as stated above.

Platelet transfusion results

A successful platelet transfusion was defined as a recovery of platelets after transfusion of 20% or more after 1 hour or 10% or more after 16 hours[17,36].

$$\% \text{ recovery} = \frac{\text{Platelet increment } (10^9/l) \text{ x blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \text{ x } 100$$

Methods used for the detection of antibodies against platelet antigens

I. Enzyme Linked Immunosorbent Assay (ELISA)

QuikScreen™ was used according to manufacturer's instruction (GTI, Brookfield, WI, USA) by incubating patient's serum with HLA class I antigens obtained from a large pool of platelets from white, black and Hispanic blood donors immobilized in a microwell strip. After adding alkaline phosphatase-conjugated goat-antihuman immunoglobulin G (IgG) and a substrate of alkaline phosphatase p-nitrophenyl phosphate (PNPP) the adsorbance was read at 405 nm. A result was considered to be positive when adsorbance exceeded the adsorbance of the negative control more than 2 times. All tests were performed in duplicate; if the results of duplicate tests differed more than 20%, the result was rejected This test detects antibodies against HLA-antigens.

II. Lymphocyte Cytotoxic Test (LCT)

After centrifuging at 1000 G (Rotixa RP 4200, Hettich, Germany) with 2.5 ml of Lymphoprep (Pocas, Oslo, Norway) and washing 2 times with 11 ml 1% bovine serum albumine in PBS (1% BSA-PBS) the mononuclear cell fraction from 5 different donors was separately suspended in 1 ml RPMI1640 (Hyclone, Logan, Utah, U.S.A.)/10% BCS (Bio Whittaker, Verviers, Belgium). Covered by a layer of Medinol oil 1 µl of this cell fraction with a concentration of 3 x 10^6/ml was incubated with 1 µl of patient serum for 30 minutes at room temperature and then 5 µl of rabbit complement was added to each well separately for the 5 different donors. After incubating for 45 minutes 2 µl of eosine was added for 3 minutes after which 10 µl of formaldehyde was added. The wells were covered and left over night at 4 ^{0}C . The next day dead cells were judged by phase contrast microscopy in triplet. If 2 or 3 of the triplet showed a more than 50% lysis the reaction against the donor was considered to be positive. If 2 or more of 5 donors showed a positive reaction the patient was considered immunized against a broad range of HLA-antigens. This test detects lymphocytotoxic antibodies, mainly against HLA-antigens.

III. Platelet and Lymphocyte Immunofluorescence Test (PIFT and LIFT)

This modified method was used as described elsewhere[37]. In short, four parameter flow cytometry was performed on a platelet and lymphocyte population from a panel of 5 donors after incubation with patient's serum and a diluted mixture of fluorescence-conjugated goat anti-IgG. A donor was determined positive when the fluoresence showed more than 17% and 36% of all platelets (PIFT) and lymphocytes (LIFT), respectively, exceeding a specific marker. A patient was considered immunized against a broad range of HLA-antigens if 40% or more of all donors showed a positive result. A result was rejected when duplo results differed more than 10%. The PIFT measures all causes of IgG bound to platelets (antibodies against HLA- and platelet-specific antigens and immune complexes). The LIFT measures mainly antibodies against HLA-antigens as previously described[38,39].

Non-immune factors

Of all transfusions the following patient related factors were scored: sex, diagnosis, therapy, splenomegaly, fever (>38.2 ^{0}C), disseminated intravascular coagulation (DIC), graft versus host disease (GvHD), Amphotericin B administration and storage time of platelet transfusions from acquisition to donation (D_{time} - A_{time}).

Statistical analysis

The endpoints of this study were platelet recovery after transfusion of 20% or more after 1 hour and 10% or more after 16 hours. The percentage of platelet transfusions with successful recovery was calculated for the different subgroups, and univariate logistic regression was used to calculate P-values for differences in recovery between the subgroups with adjustment for multiple transfusions in single patients. All P-values are two-sided and P-values ≤ 0.05 were considered statistically significant.

Results

Immune factors

A total of 181 platelet transfusions were studied in 97 patients. Test results were complete 181 times (100%) in the PIFT and LIFT, 178 times (98%) in the LCT, because of technical failure, and 175 times (97%) in the ELISA, because of more than 20% variation in duplo results. A recovery of less than 20% after 1 hour or less than 10% after 16 hours was seen in 72 (40%) and 67 (37%) transfusions, respectively. The recovery after 1 hour of less than 20% showed a positive test result of the ELISA in 12 (17%), LCT in 5 (7%), LIFT in 12 (17%) en PIFT in 8 (11%) sera (Table 2A).

Table 2. Univariate logistic regression of recovery ≥ 20% at 1 hour (IIA) and recovery ≥ 10% at 16 hours (IIB) for all 4 tests (ELISA, LCT, LIFT and PIFT) with adjustment for multiple transfusions in single patients

a.

	Total *n*	Recovery ≥ 20% *n* (%)	OR (95%CI)	P-value
ELISA				0.24
Negative	151	93 (62%)	1	
Positive	24	12 (50%)	0.6 (0.3-1.4)	
LCT				0.45
Negative	162	95 (59%)	1	
Positive	16	11 (69%)	1.6 (0.5-4.8)	
LIFT				0.98
Negative	151	91 (60%)	1	
Positive	30	18 (60%)	1.0 (0.5-2.1)	
PIFT				0.46
Negative	164	100 (61%)	1	
Positive	17	9 (53%)	0.7 (0.3-1.7)	

b.

	Total *n*	Recovery ≥ 10% *n* (%)	OR (95%CI)	P-value
ELISA				0.65
Negative	151	95 (63%)	1	
Positive	24	14 (58%)	0.8 (0.4-1.9)	
LCT				0.12
Negative	162	99 (61%)	1	
Positive	16	13 (81%)	2.8 (0.8-9.9)	
LIFT				0.28
Negative	151	98 (65%)	1	
Positive	30	16 (53%)	0.6 (0.3-1.5)	
PIFT				0.06
Negative	164	107 (65%)	1	
Positive	17	7 (41%)	0.4 (0.1-1.0)	

n = number of transfusions; OR = odds ratio; 95%CI = 95% confidence interval

The recovery after 16 hours of less than 10% showed a positive ELISA in 10 (15%), LCT in 3 (5%), LIFT in 14 (21%) and PIFT in 10 (15%) sera (Table 2B). No statistically significant correlation between test results and recovery after 1 or 16 hours could be demonstrated (Table IIA and IIB).

Positive tests when recovery after 1 hour was more than 20%, was found in 12 (11%), 11 (10%), 18 (17%) and 9 (8%) sera in ELISA, LCT, LIFT and PIFT respectively and after 16 hours in 14 (13%), 13 (12%), 16 (14%) and 7 (6%) sera. In order to increase the specificity of tests a combination of HLA-antibody measuring tests (ELISA, LCT and LIFT) and PIFT was investigated. No correlation was found between a recovery after 1 hour of less than 20% and a positive ELISA, LCT or LIFT in combination with a positive PIFT. The only statistically significant association was demonstrated between a recovery after 16 hour of less than 10% and a positive ELISA and PIFT, $P = 0.04$, or a positive LIFT and PIFT, $P = 0.03$.

In 124 transfusions (69%), in which all tests were found to be negative, a recovery of less than 20% after 1 hour and less than 10% after 16 hours was found in 50 (40%) and 50 (40%), respectively. In 43 (86%) of the 50 transfusions with negative tests and low recovery after 1 hour one or more non-immunological causes of low recovery (splenomegaly, fever, Amphotericine B administration, DIC, more than 3 days between platelet acquisition and donation) were present. In 39 (78%) of the 50 transfusions with negative tests and low recovery after 16 hour one or more non-immunological causes of low recovery were present.

Non-immune factors

Several non-immune factors showed an association with a low platelet recovery (Table 3A and IIIB).

Splenomegaly showed a correlation with low platelet recovery both 1 hour ($P < 0.01$) and 16 hours ($P = 0.02$) after platelet transfusion. Presence of CML diagnosis also correlated with a low platelet recovery after 1 hour ($P < 0.01$) and 16 hours ($P = 0.02$) when compared with AML diagnosis. However, in 15 of 21 transfusions in patients with CML splenomegaly was present. Allogeneic stem cell transplantation correlated well with a low platelet recovery after 1 hour ($P = 0.01$) and 16 hours ($P = 0.05$). Also in 12 of 16 transfusions in patients with allogeneic stem cell transplantation splenomegaly was present. Female sex correlated with high platelet recovery after 1 hour ($P = 0.05$) but not after 16 hours ($P = 0.68$). Further study of this issue showed that splenomegaly was almost solely present in male patients (44 male patients vs. 8 female patients). When patients with splenomegaly were left out of the analysis no association could be demonstrated between female sex and high platelet recovery after 1 hour anymore. Longer storage time of platelet concentrates, especially when platelet concentrates were

stored more than 3 days, showed a low platelet recovery after 1 hour (P = 0.01) and after 16 hours (P = 0.03). Administration of Amphotericin B was associated with a high platelet recovery after 1 hour (P = 0.05) but not after 16 hours (P = 0.87). Also other therapies, donor lymphocyte infusion (DLI), canine antithymocyte globulin (ATG) or other immunotherapy, showed a correlation after 1 hour with low platelet recovery (P = 0.04), but this was not observed after 16 hours (P = 0.10). Other non-immune factors we studied (fever, DIC and GvHD) showed no correlation with platelet recovery.

Table 3. Univariate logistic regression of recovery ≥ 20% at 1 hour (IIIA) and recovery ≥ 10% at 16 hours (IIIB) for non-immunological factors (fever, DIC and GvHD were left out because no significant association was shown) with adjustment for multiple transfusions in single patients

a.

Item	*Total* *n*	Recovery ≥ 20% *n* (%)	OR (95%CI)	P-value
Sex				0.05
Male	112	60 (54%)	1	
Female	69	49 (71%)	2.1 (1.0-4.5)	
Diagnosis[1]				0.006
AML	91	60 (66%)	1	
ALL	8	3 (38%)	0.3 (0.1-1.5)	
CML	21	5 (24%)	0.2 (0.1-0.4)	
MM	6	5 (83%)	2.6 (0.3-23.5)	
NHL	23	17 (74%)	1.5 (0.5-4.6)	
HD	5	3 (60%)	0.8 (0.1-6.2)	
MDS	20	13 (65%)	1.0 (0.2-4.2)	
Other	6	3 (50%)	0.5 (0.2-1.5)	
Therapy[2]				0.004
Chemo	102	70 (69%)	1	
AutoBMT	14	12 (86%)	2.7 (0.5-13.7)	
AlloBMT	59	26 (44%)	0.4 (0.2-0.8)	
Other therapy	6	1 (17%)	0.1 (0.0-0.9)	
Splenomegaly				0.005
No	129	88 (68%)	1	-
Yes	52	21 (40%)	0.3 (0.1-0.7)	
Amphotericin B				0.05
No	164	95 (58%)	1	
Yes	17	14 (82%)	3.4 (1.0-11.5)	
$\mathbf{D_{time} - A_{time}}$[3]				0.01
≤ 3 days	93	64 (69%)	1	
> 3 days	85	44 (52%)	0.5 (0.3-0.9)	

b.

Item	Total *n*	Recovery ≥ 10% *n* (%)	OR (95%CI)	P-value
Sex				0.68
Male	112	69 (62%)	1	
Female	69	45 (65%)	1.2 (0.6-2.5)	
Diagnosis[1]				0.23
AML	91	61 (67%)	1	
ALL	8	6 (75%)	1.5 (0.2-11.2)	
CML	21	7 (33%)	0.2 (0.1-0.8)	
MM	6	5 (83%)	2.5 (0.3-22.5)	
NHL	23	17 (74%)	1.4 (0.4-4.6)	
HD	5	2 (40%)	0.3 (0.0-2.6)	
MDS	20	12 (60%)	0.7 (0.2-2.4)	
Other	6	3 (50%)	0.5 (0.2-1.4)	
Therapy[2]				0.11
Chemo	102	72 (71%)	1	
AutoBMT	14	9 (64%)	0.8 (0.2-2.9)	
AlloBMT	59	31 (53%)	0.5 (0.2-1.0)	
Other therapy	6	2 (33%)	0.2 (0.0-1.4)	
Splenomegaly				0.02
No	129	89 (69%)	1	
Yes	52	25 (48%)	0.4 (0.2-0.9)	
Amphotericin B				0.87
No	164	103 (63%)	1	
Yes	17	11 (65%)	1.1 (0.4-2.9)	
$\mathbf{D_{time} - A_{time}}$[3]				0.03
≤ 3 days	93	65 (70%)	1	
> 3 days	85	47 (55%)	0.5 (0.3-0.9)	

n = number of transfusions with specified test results; OR = odds ratio; 95%CI = 95% confidence interval

[1] Diagnoses are compared to recovery of platelet transfusions in patients with AML; CLL is not shown because only 1 platelet transfusion was executed in the patient with CLL.

[2] Therapies are compared to recovery of platelet transfusions in patients with chemotherapy.

[3] Time between acquisition of the donor platelets and donation of the platelet transfusion.

In 42 transfusions (23%), in which no non-immune factors were present, a platelet recovery of less than 20% after 1 hour and less than 10% after 16 hours were found in 8 (19%) and 11 (26%), respectively. In the 8 transfusions with no non-immune factors and low platelet recovery after 1 hour ELISA, LCT, LIFT and PIFT were positive in 0, 1, 0 and 0 sera, respectively. In the 11 transfusions with no non-immune factors and low platelet recovery after 16 hour no positive tests were found.

Discussion

In our study in 97 patients with hematological diseases about 40% of platelet transfusions resulted in a low platelet recovery, which is in agreement with literature[8,9,11,13,16,17,21]. Alloimmunisation, defined as positivity in one or more tests, could be demonstrated in 5-21% of platelet transfusions with a low recovery after 1 or 16 hours. This low percentage was expected because of a low prevalence of alloimmunisation in the patient population we studied. The low prevalence of alloimmunisation was caused by the absence of selection of patients, and the leucodepletion of the transfusions. In our study only 10 of 97 patients were measured more than 3 times. Other studies, that show data from sometimes highly selected patients[21,30] over longer periods of time[15,18,32], demonstrate higher rates of alloimmunisation. The usage of leucodepleted blood products also diminishes the prevalence of alloimmunisation[24,25] in contrast with the usage of non-leucodepleted blood products used in most published studies[3–9,11,13,26,28,29]. Also difference in the definition of alloimmunisation may play a role.

No clear correlation could be demonstrated between alloimmunisation demonstrated by positivity of a single test and low recovery of a subsequent platelet transfusions after 1 and 16 hours. On the other hand we showed that alloimmunisation was present in 6-14% of platelet transfusions with a good recovery. This discrepancy can be caused by the inability of the immunoglobulins, that cause the positive test results, to activate the complement or bind to the macrophage system in these patients. Also immunoglobulins with restricted specificity may cause positive test results against panel cells but do not react with the platelets from the 5 different donors of which the transfusion exists. Otherwise in the 11 platelet transfusions with a low recovery and no non-immune causes alloimmunisation could be demonstrated in only 1, leaving 10 platelet transfusions without explanation. This may be explained by other non-immune causes of low platelet recovery we did not measure in this study or by a lack of sensitivity in the tests we studied. In the literature LCT is the most studied test in demonstrating alloimmunisation showing a good correlation with low platelet recovery[6,8,26]. The LCT, however, in our hands is less sensitive than the ELISA and LIFT, the correlation between these two tests

being high[25]. Other tests that have demonstrated a correlation with platelet recovery are PIFT[7], ^{51}Cr platelet lysis assay[27] and ELISA[18]. These studies however were not designed to demonstrate a correlation between a single test and recovery in a subsequent platelet transfusion in a non-selected patient population using leucodepleted blood products.
Combining the ELISA with PIFT or LIFT with PIFT did demonstrate a correlation with recovery of a subsequent platelet transfusions after 16 hours, but not after 1 hour. It seems unlikely that the presence of antibodies against platelet specific antigens next to anti-HLA antibodies are resposible for this finding. Positivity in two tests, one measuring HLA-antibodies and the other HLA-antibodies and platelet specific antibodies, increases specificity and in that way diminishes false positive results. In most studies several combination of tests have demonstrated a correlation between platelet refractoriness and alloimmunisation[9,11,13–15,29,30,32]. However these studies did not use leukodepleted platelet transfusions or were not designed to demonstrate a correlation with a subsequent platelet transfusion.
Of the non-immune factors splenomegaly, male sex, CML, allogeneic stem cell transplantation and other therapies, DLI, ATG or other immunotherapy, were found to have a negative effect on platelet recovery after 1 hour in a univariate analysis. Low platelet recovery after 16 hours was correlated with splenomegaly, storage time of more than 3 days, CML and allogeneic stem cell transplantation. However further investigation showed splenomegaly to be a confounding factor in sex, CML and allogeneic stem cell transplantation. A correlation of platelet recovery with CML and allogeneic stem cell transplantation were also found by others[9,11,15,21] but no comment was made as towards splenomegaly as a confounding factor.
Immune causes of platelet refractoriness are infrequently encountered in a non-selected patient population receiving leucodepleted transfusions. Randomly executed tests, that detect alloantibodies, are not predictive for platelet recovery in these patients. Non-immune causes, known to influence platelet recovery, are frequently present, of which splenomegaly and a storage time of more than 3 days are the main causes of a low platelet recovery.

Acknowledgements

We thank Anneke Luijten and Marianne Beije for their help in collecting sera and data. We thank Nicole Bakker for her help on performing the tests and data collection and storing. We thank Dew Doekharan for his help with the data management.

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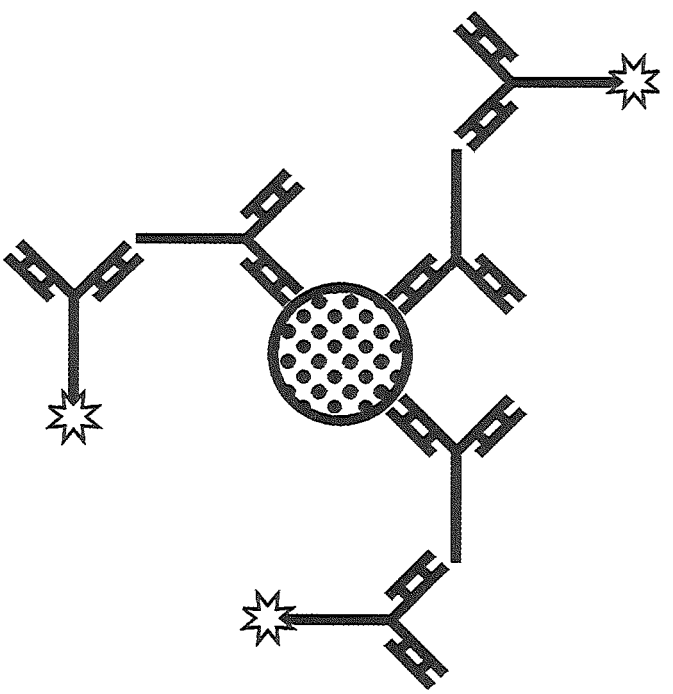
Chapter 7

The relation of crossmatch tests and screening tests and their predictive value for platelet recovery after transfusion



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Abstract

Background and Objectives

Alloantibodies against platelets can be detected by different laboratory tests. Most of these tests use panel cells or antigens as a target. In relation to these tests a crossmatch test of transfused platelets and patient's serum may be viewed of as the standard for detecting donor platelet destruction by alloimmunization.

Methods and materials

In 95 randomly selected thrombocytopenic patients with hematological malignancies receiving leukodepleted blood products 184 serum samples were studied in an *in vitro* crossmatch test according to the technique of the platelet immunofluorescence test (crossmatch-PIFT), in an *in vivo* crossmatch detecting binding of immunoglobulins *in vivo* to transfused platelets (*in vivo*-PIFT), in the indirect PIFT using 5 random donors as a target (panel-PIFT) and in an enzyme linked immunosorbent assay using immobilized HLA-antigens of 100 standardised donors (ELIHLA). The results of all these methods were related to the recovery at 1 and 16 hours post transfusion.

Results

The results of the crossmatch-PIFT were not associated with platelet recovery at 1 and 16 hours after transfusion. Even in a patient subgroup, in which predefined clinical factors were excluded, no association with platelet recovery was found. The results of the crossmatch-PIFT correlated with those of the *in vivo*-PIFT ($P = 0.02$), however, 35 (19%) discrepant results were established between these tests. The results of the crossmatch-PIFT were not related to the panel-PIFT ($P = 0.25$), but did relate to those of the ELIHLA ($P = 0.02$) still revealing 36 (20%) discrepant results. Neither the *in vivo*-PIFT, the panel-PIFT nor the ELIHLA was associated with platelet recovery after 1 hour, whilst only a positive panel-PIFT was associated with poor platelet recovery at 16 hours after transfusion ($P = 0.03$).

Conclusion

In a population at low risk for alloimmunization the correlation of test outcome and platelet recovery is poor. None of the tests, crossmatch tests nor screening tests, showed to be superior in this population.

Introduction

Patients with hematological malignancies who receive intensified chemotherapy often need platelet support to prevent bleeding complications. Twenty-four to 44% of platelet transfusions fail to produce satisfactory responses because of non-immune factors (63-67%), immune factors (18-25%) or both[1-4]. In order to distinguish immune from non-immune factors different serological tests are routinely used. Examples of these tests are lymphocytotoxicity (LCT)[5-7], immunofluorescence (IFT)[8-13], enzyme-linked immunosorbent assays (ELISA)[14-17], or monoclonal antibody-specific immobilization of platelet antigens assays (MAIPA)[18–20]. In all these tests patient's serum is incubated with donor panel cells as a target in order to demonstrate the presence of alloimmunization. More recently other techniques have been described, which use a broad range of readily available HLA-antigens as a target. Examples of the latter tests are flow cytometry of latex beads coated with HLA class I antigens[21,22] and an ELISA tests with a broad range of immobilized HLA class I antigens (ELIHLA)[23-27]. We recently demonstrated the limited correlation of tests using donor panel cell or antigens, such as the LCT, platelet immunofluorescence test (PIFT), the lymphocyte immunofluorescence test (LIFT) and the ELIHLA, with the outcome of platelet transfusions in patients receiving leukodepleted platelet transfusions[27]. For this reason we developed a test that measured physiologic circumstances of *in vivo* binding of antibodies to transfused platelets, the *in vivo* binding of immunoglobulin G (IgG) in the platelet immunofluorescence test (IVBI-PIFT, renamed *in vivo*-PIFT for clarity in this study)[28]. This test can be considered as an *in vivo* crossmatch test.

In view of these data the question arises if there is superiority *in vitro* crossmatch testing in relation to *in vivo* crossmatch testing (*in vivo*-PIFT) and screening tests using panel cells (panel-PIFT and ELIHLA) in predicting transfusion outcome. In this way the importance of difference in target cells and in *in vitro* or *in vivo* binding of immunoglobulins to transfused platelets was assessed in relation to test results and to platelet transfusion outcome.

Methods and Materials

Serum samples

We studied 184 serum samples from 95 randomly selected patients, mean 1.9 transfusions per patient (range 1-8), who were treated for a malignant lymphoma or leukemia in our hospital. One patient was treated with rabbit antithymocyte globulins; none with human intravenous immunoglobulins. Patients received a leukocyte

depleted platelet transfusion, a suspension of 5 random ABO-matched donors, (i) prophylacticly when platelet count was less than 10 x 10^9/l, (ii) before an intervention or because of clinical manifest bleeding at higher platelet counts. The serum samples were collected before and 1 and 16 hours after platelet transfusion. The median platelet count before platelet transfusion was 11 x 10^9/l (range 4-60 x 10^9/l).

Platelet concentrates

Platelet concentrates were obtained from the Sanquin Blood Bank South West Region (Rotterdam, the Netherlands) and were prepared from the blood of 5 HLA-unmatched ABO-matched volunteer donors. The average content of the platelet concentrates was 4 x 10^{11} platelets in 250 ml. The platelet concentrates were filtered by a PLX-5 Asahi filter (Baxter, La Chatre, France) to deplete the transfusion from leukocytes. The average leukocyte number was 0.05 x 10^6 per platelet concentrate (range 0.00-0.20 x 10^6). In patients receiving a stem cell transplantation the platelet concentrates were irradiated with 25 Gy in order to prevent graft versus host disease (GvHD) by transfused lymphocytes[29]. The platelet concentrates were stored at 22 ^{0}C until used for transfusion. Also erythrocyte concentrates were always leukocyte depleted and for the same reasons irradiated as mentioned above.

Alloantibody detecting tests

Crossmatch-PIFT

The crossmatch-PIFT was performed according to a modified method of the indirect PIFT, as described elswhere[11, 13, 28], and the transfused platelets as target antigens. From the platelet transfusion bag 100 µl of suspension was washed twice with a buffer, 10 g ethyleendiamine-tetra-acetate (EDTA), 8.5 g NaCl, 1.61 g $Na_2HPO_4.2H_2O$ and 0.2 g $NaH_2PO_4.2H_2O$ in 1 liter Millipore H_2O, titrated up to pH = 7.35 with 10 mmol NaOH and mixed with 9 ml bovine serum albumin 22%, (EDTA-PBS 0.2% BSA) at 1800 G for 10 minutes. After removing the supernatant the cell pellet was resuspended to a platelet suspension of 100 x 10^6 cells/ml. To 50 µl of the platelet suspension 50 µl of serum was added and incubated at room temperature in the dark for 30 minutes. After washing for 3 times with 850 µl EDTA-PBS 0.2% BSA at 1800 G for 5 minutes, 50 µl of fluorescence-conjugated goat anti-human IgG (FITC) (Nordic, Tilburg, The Netherlands) was added and incubated at room temperature in the dark for 30 minutes. After washing for 3 times with 850 µl EDTA-PBS 0.2% BSA at 1800 G for 5 minutes 10 µl LDS-PI (5 µl LDS stock [0.2 mg laser dye stock solution 751 in 1 ml methanol] (Applied Laser Technology, Maarheze, the Netherlands) together with 5 µl PI stock [4 mg propidium iodide in 1 ml PBS] (Sigma Chemicals, St

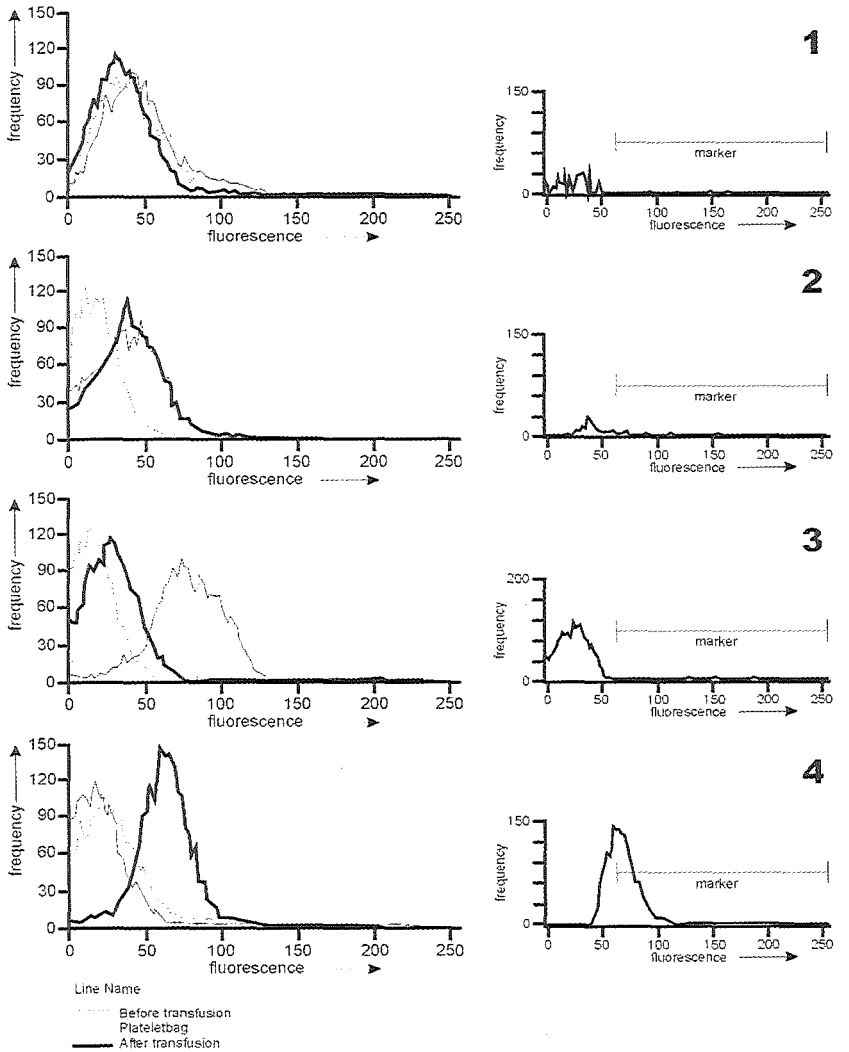
Louis, MO, USA) in 10 ml EDTA-PBS 0.2% BSA) was added to the suspension and incubated for 15 minutes.
After adding 50 µl of EDTA-PBS 0.2% BSA 4-parameter flow cytometry was performed collecting 5,000 events, using a live gate to select the platelets. In this way a histogram was made that showed the FITC-fluorescence on a logarithmic scale. Of every studied platelet transfusion the platelets were also incubated with previously defined negative and positive sera. The average percentage of platelets from the negative control exceeding a predefined fluorescence marker in 100 samples was 15% with a standard deviation of 11%. We subtracted this average plus 1 standard deviation, together 26%, from the percentage platelets exceeding the predefined marker in the tested serum sample. All tests were performed in duplicate, so that the final crossmatch-PIFT results were generated using the average percentage of platelets exceeding the predefined marker of these 2 duplicate results corrected by the average plus 1 standard deviation from the negative control (26% in this series). The crossmatch-PIFT detects most causes of IgG binding to platelets (antibodies against HLA-class I antigens, against platelet specific antigens and autoantibodies).

In vivo-PIFT

The *in vivo*-PIFT was executed by collecting blood before and one hour after platelet transfusion in duplicate according to the technique of the *In Vivo* Binding of Immunoglobulins in the Platelet Immunofluorescence Test (IVBI-PIFT)[28]. After several centrifuging steps, as described elswhere[28], three platelet suspensions were used: (I) from the blood drawn before transfusion, (II) from the platelets out of the transfusion bag as an internal control and (III) from the blood drawn after transfusion. These three suspensions were separately incubated with a diluted mixture of fluorescence-conjugated goat anti-human IgG (FITC) (Nordic, Tilburg, The Netherlands), after which 4-parameter flow cytometry was performed acquiring 8,000 platelets per suspension. An overlay histogram was generated from the list mode data on FITC fluorescence of platelet suspensions I, II and III. This combined histogram was used for visual scoring. These scores were categorised as pattern 1 (no alloimmunization), pattern 2 (binding of IgG not related to alloimmunization), pattern 3 (preferential binding of IgG to own or previously transfused platelets) and pattern 4 (alloimmunization) (Figure 1). Thus, pattern 4 was interpreted as positive, indicating alloimmunization, while patterns 1, 2 and 3 were classified as no alloimmunization. More standardized results of the *in vivo*-PIFT could be generated by a histogram subtraction method [29]. The histograms of the IgG-fluorescence of suspension I (prior to transfusion) was subtracted from the histogram of suspension III (post transfusion). This was done by subtracting the number of platelets for each fluorescence channel

separately (linear scale of 256 histogram channels). If differences between the corresponding channels of histogram I and III resulted in negative values, these were replaced by zero events for those channels in the subtraction histogram (Figure 1).

Figure 1. Results of the *in vivo*-PIFT according to the way these results were generated



The left panels show the visual scoring method of the in vivo-PIFT, demonstrating the 4 different patterns. The right panels show the subtraction analysis of histograms, the positive cumulative events of the events after platelet transfusion minus the events before platelet transfusion per fluorescence channel.

Histogram II was used as internal control. This leaves a subtraction histogram that can be characterised by (i) the number of remaining events (= total number of platelets of all fluorescence channels after this subtraction) and (ii) the mean fluorescence of the remaining events. First, *"Events post subtraction"*(EPS) was defined as the average of all remaining events of both (duplicate) subtraction histograms. Second, *"Mean channel post subtraction"* (MCPS) was defined as the average of the mean channel values of both (duplicate) subtraction histograms (Figure 1). Finally, results of this subtraction were defined as positive when EPS $\geq$ 1100 and MCPS $\geq$ 30; all other results were classified negative. The *in vivo*-PIFT detects all causes of IgG binding to platelets (antibodies against HLA-class I antigens, against platelet specific antigens, autoantibodies and immunecomplexes).

Panel-PIFT

This assay was performed as a modified method from a PIFT assay described elsewhere[28]. In short, 4-parameter flow cytometry was performed after incubating patient's serum with platelets from 5 random donors separately, obtained from the Sanquin Blood Bank South West Region, followed by a second incubation with a diluted mixture of fluorescence-conjugated goat anti-human IgG. The platelets from the 5 random donors were collected the day before, separated from other blood components and washed two times. A panel cell suspension was considered to be positive when the fluoresence demonstrated more than 17% of all platelets exceeding predefined fluorescence marker (previously determined by more than 100 negative controls)[11,27]. The final results of the panel-PIFT were generated as the average percentage of positive donors of duplicate tests. A patient was considered immunized if 40% or more of all donors showed a positive result. The panel-PIFT measures most causes of IgG bound to platelets (antibodies against HLA- and platelet-specific antigens and autoantibodies).

Enzyme Linked Immunosorbent Assay (ELIHLA)

QuikScreen™ was used according to manufacturer's instructions (GTI, Brookfield, WI, USA) by incubating patient's serum with HLA class I antigens obtained from a platelet pool of about 100 donors consisting of white, black and Hispanic blood donors, immobilized in a microwell strip[10]. After adding alkaline phosphatase-conjugated goat-antihuman immunoglobulin G (IgG) and a substrate of alkaline phosphatase p-nitrophenyl phosphate (PNPP) the adsorbance was read at 405 nm. Final results were generated by the average of duplicate results. A result was considered to be positive when adsorbance exceeded the adsorbance of the negative

control more than 2 times. This ELISA detects antibodies of the IgG-class against HLA-class I antigens.

Platelet transfusion results

A successful platelet transfusion was defined as a recovery of platelets after transfusion of (i) 20% or more after 1 hour or (ii) 10% or more after 16 hours[31-33] according to the following formula:

$$\% \text{ recovery} = \frac{\text{Platelet increment } (10^9/l) \text{ x blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \text{ x } 100$$

Non-immune factors

For every platelet transfusion the presence of non-immune factors affecting survival of transfused platelets was investigated (diagnosis, therapy, splenomegaly, fever (>38.2 ^{0}C), disseminated intravascular coagulation, graft versus host disease, Amphotericin B administration and storage time of platelet transfusions from acquisition to donation). Previously, we have demonstrated in the same patient population that only the presence of splenomegaly and a platelet transfusion storage time of more than 3 days correlated with poor platelet recovery[33]. We performed a subgroup analaysis in transfusions with the presence of the latter two non-immunological factors.

Statistical methods

Results of the crossmatch-PIFT were compared with the results of the *in vivo*-PIFT using Pearson's chi-squared test, while Fisher's exact test was used to compare the results of the crossmatch-PIFT with the results of the panel-PIFT and ELIHLA. Univariate logistic regression with adjustment for patients with multiple transfusions was used to investigate the association between platelet recovery after 1 and 16 hours and the results of the *in vivo*-PIFT, the crossmatch, the panel-PIFT and the ELIHLA. All P-values are two-sided and P-values ≤ 0.05 were considered statistically significant.

Results

Crossmatch-PIFT and platelet recovery

In the 184 studied serum samples the crossmatch-PIFT showed a positive result in 26 (14%) and a negative result in 158 (86%). Of the transfusions with a positive crossmatch-PIFT a poor platelet recovery was found in 14 (54%) and 12 (46%) at 1 and 16 hours after transfusion, respectively. The transfusions with a negative crossmatch-PIFT demonstrated a poor recovery in 63 (40%) and 53 (36%, in 11 transfusions no recovery after 16 hours was available) transfusions at 1 and 16 hours after transfusion. A positive crossmatch-PIFT did not predict a poor platelet recovery neither after 1 hour (odds ratio [OR] = 0.57, 95% confidence interval [CI] = 0.23-1.38, P = 0.21) nor after 16 hours (OR = 0.66, 95% CI = 0.28-1.54, P = 0.33).

Of 67 platelet transfusions without the presence of previously determined non-immune factors that correlated with poor platelet recovery (i.e., splenomegaly and storage time of platelets more than 3 days)[33] 17 (25%) platelet transfusions demonstrated a recovery of less than 20% after 1 hour. In these 17 platelet transfusions a positive crossmatch-PIFT was demonstrated in only 3 platelet transfusions (18%). Next to this, 15 transfusions (22%) demonstrated a recovery of less than 10% after 16 hours in the 67 transfusions without predetermined non-immune factors. A positive crossmatch-PIFT was demonstrated in 1 transfusion (7%) of these 15 transfusions. A positive crossmatch-PIFT did not predict a poor platelet recovery in this subgroup analysis neither after 1 hour (OR = 0.64, 95% confidence interval [CI] = 0.14-2.93, P = 0.56) nor after 16 hours (OR = 2.73, 95% CI = 0.29-26.1, P = 0.38).

Crossmatch-PIFT versus *in vivo*-PIFT

The difference in *in vitro* or *in vivo* binding of immunoglobulins was studied in the 184 transfusions by relating the results of the crossmatch-PIFT to those of the in vivo-PIFT. The results of the crossmatch-PIFT corresponded well with the *in vivo*-PIFT (P = 0.02) (Table 1).

Table 1. The results of the crossmatch-PIFT related to the results of the *in vivo*-PIFT, the panel-PIFT and the ELIHLA

	Crossmatch-PIFT		*Total*
	Negative	**Positive**	
	***n* = 158**	***n* = 26**	***n* = 184**
***in vivo*-PIFT**[†]			
Negative	142	19	161
Positive	16	7	23
Panel-PIFT[‡]			
Negative	146	22	168
Positive	12	4	16
ELIHLA[*]			
Negative	141	19	160
Positive	17	7	24

[†] *P = 0.02*
[‡] *P = 0.25*
[*] *P = 0.02*

Nevertheless the in vivo-PIFT was positive in 16 (9%) transfusions in which the crossmatch-PIFT was negative, whereas the crossmatch-PIFT was positive in 19 (10%) transfusions in which the *in vivo*-PIFT was negative.
In 7 transfusions with a positive result of both *in vivo*-PIFT and crossmatch-PIFT a poor platelet recovery after 1 and 16 hours was found in 4 and 3 of transfusions, respectively (Table 2).

Table 2. Studied serum samples divided into 4 groups depending on positive or negative results of crossmatch-PIFT and *in vivo*-PIFT related to the platelet recovery after 1 and 16 hours of the studied platelet transfusion

Crossmatch-PIFT	***in vivo*-PIFT**	***Total***	**Recovery$_{1h}$ < 20%**	**Recovery$_{16h}$ < 10%**
		n	***n* (%)**	***n* (%)**
Positive	Positive	7	4 (57%)	3 (43%)
Positive	Negative	19	10 (53%)	9 (47%)
Negative	Positive	16	4 (25%)	2 (13%)[†]
Negative	Negative	142	59 (42%)	51 (39%)[‡]

[†] *1 recovery after 16 hours was not known*
[‡] *10 recoveries after 16 hour were not known*

In the 19 transfusions with a negative *in vivo*-PIFT and a positive crossmatch-PIFT a poor platelet recovery was observed in 10 and 9 of transfusions after 1 and 16 hours,

respectively. In 16 transfusions with a positive *in vivo*-PIFT and a negative crossmatch-PIFT a poor platelet recovery was demonstrated in 4 and 2 of transfusions after 1 and 16 hours, respectively. Finally, in the 142 transfusions with a negative *in vivo*-PIFT and a negative crossmatch-PIFT a poor platelet recovery was found in 59 (42%) and 51 (39% of 132) of transfusions after 1 and 16 hours. Overall, the *in vivo*-PIFT did not predict a poor platelet recovery at 1 hour (OR = 1.41, 95% CI = 0.55-3.59, P = 0.48) nor at 16 hours (OR = 2.24, 95% CI = 0.77-6.54, P = 0.14) after platelet transfusion.

Crossmatch-PIFT versus panel-PIFT

The comparison between crossmatch testing and screening tests was studied by relating the results of the crossmatch-PIFT to those of the panel-PIFT in the 184 studied platelet transfusions. The results of the crossmatch-PIFT corresponded poorly with those of the panel-PIFT (P = 0.25) (Table 1). In 12 transfusions the crossmatch-PIFT was negative and the panel-PIFT positive, whilst the crossmatch-PIFT was positive and the panel-PIFT negative in 22; a total of 34 (18%) discrepant results. A positive panel-PIFT did not predict a poor recovery after 1 hour (OR = 0.70, 95% CI = 0.29-1.65, P = 0.41) either, but had some predictive power for poor platelet recovery after 16 hours (OR = 0.32, 95% CI = 0.12-0.89, P = 0.03). In 4 transfusions, in which both crossmatch-PIFT and panel-PIFT showed positive results, 3 and 4 of transfusions demonstrated poor recoveries after 1 and 16 hours, respectively (Table 3).

Table 3. Studied serum samples divided into 4 groups depending on positive or negative results of crossmatch-PIFT and panel-PIFT related to a poor platelet transfusion result after 1 and 16 hours

Crossmatch-PIFT	**panel-PIFT**	***Total***	**$Recovery_{1h} < 20\%$**	**$Recovery_{16h} < 10\%$**
		n	***n* (%)**	***n* (%)**
Positive	Positive	4	3 (75%)	4 (100%)
Positive	Negative	22	11 (50%)	8 (36%)
Negative	Positive	12	5 (42%)	6 (50%)
Negative	Negative	146	58 (40%)	47 (35%)[†]

[†] *of 11 transfusions no recovery was measured after 16 hours*

In the 22 transfusions with a positive crossmatch-PIFT and a negative panel-PIFT poor platelet recovery after 1 and 16 hours was demonstrated in 11 and 8 of transfusions, respectively. Of the 12 transfusions with a negative crossmatch-PIFT and a positive panel-PIFT 5 and 6 demonstrated a poor platelet recovery after 1 and 16 hours, respectively. Finally, the 146 transfusions with negative test results in both

crossmatch-PIFT and panel-PIFT were followed by poor platelet recoveries after 1 and 16 hours in 58 (40%) and 47 (35% of 135) of transfusions, respectively.

Crossmatch-PIFT versus ELIHLA

In order to study whether difference with crossmatch testing could be minimized by using a screening test with a larger number of HLA-antigens as a target, we related the results of the crossmatch-PIFT with those of the ELIHLA in 184 transfusions. There was a statistically significant association between crossmatch-PIFT and ELIHLA result (P = 0.02) (Table 1). However, in 17 transfusions the crossmatch-PIFT was negative and the ELIHLA positive, whereas the crossmatch-PIFT was positive and the ELIHLA negative in 19; a total of 36 (20%) discrepant results. A positive ELIHLA was not associated with a poor recovery, neither after 1 hour (OR = 0.68, 95% CI = 0.31-1.49, P = 0.34) nor after 16 hours (OR = 0.75, 95% CI = 0.33-1.74, P = 0.51). In 7 transfusions, in which both crossmatch-PIFT and ELIHLA showed positive results, 5 and 3 of transfusions showed poor transfusions results after 1 and 16 hours, respectively (Table 4).

Table 4. Studied serum samples divided into 4 groups depending on positive or negative results of crossmatch-PIFT and ELIHLA related to a poor platelet transfusion result after 1 and 16 hours

Crossmatch-PIFT	ELIHLA	*Total* *n*	Recovery$_{1h}$ < 20% *n* (%)	Recovery$_{16h}$ < 10% *n* (%)
Positive	Positive	7	5 (71%)	3 (43%)
Positive	Negative	19	9 (47%)	9 (47%)
Negative	Positive	17	7 (41%)	7 (44%)[†]
Negative	Negative	141	56 (40%)	46 (35%)[‡]

[†] *of 1 transfusion no recovery was measured after 16 hours*

[‡] *of 10 transfusions no recovery was measured after 16 hours*

In the 19 transfusions with a positive crossmatch-PIFT and a negative ELIHLA poor platelet recoveries after both 1 and 16 hours were observed in 9 of transfusions. Of the 17 transfusions with a negative crossmatch-PIFT and a positive ELIHLA a poor platelet recovery after 1 and 16 hours was demonstrated in 7 and 7 (of 16 transfusions) of transfusions, respectively. Finally, of the 141 transfusions with negative test results in both crossmatch-PIFT and ELIHLA a poor platelet recovery after 1 and 16 hours was found in 56 (40%) and 46 (35% of 131) of transfusions, respectively.

Discussion

We have reported that the results of widely used screening tests that detect antibodies against platelets, do not correlate with the recovery of donor platelets in a non-selected patient population receiving leukodepleted blood products[34]. Not only can positive test results be seen in platelet transfusions with good recoveries but also negative test results can be encountered in platelet transfusions with poor recoveries even in the absence of non-immune factors that may jeopardize platelet recovery.

In order to clarify the lack of correlation between alloantibody tests and platelet recovery, we studied the relation between the crossmatch-PIFT and the *in vivo*-PIFT (previously named IVBI-PIFT)[28]. Possible differences of *in vivo* and *in vitro* binding of IgG to platelets are: (I) differences in the concentration of the alloantibodies; (II) differences in temperature during incubation (37 ^{0}C versus room temperature); (III) presence of interfering proteins in the serum; (IV) presence of non-specific binding of immunoglobulins such as immune complexes (V); length of incubation (*in vitro* 30 minutes versus 1 hour *in vivo*); and (VI) the removal of alloantibody-coated platelets from the circulation. We demonstrated that the results of *in vitro* and *in vivo* incubation of the platelets were significantly associated (P = 0.02), although discrepant results were observed in 36 (20%) of cases. In order to study whether the results of the *in vivo* binding test might be more predictive than those of *in vitro* binding tests for platelet recovery, we performed subset analysis on the 4 groups as defined by the results of *in vivo* and *in vitro* assays. In the 7 transfusions with positive results for both *in vivo* and *in vitro* incubations about half demonstrated a poor recovery after 1 and 16 hours. Not all transfusions demonstrated a poor recovery, which may be caused by a density of alloantibodies too low to trigger physiological destruction mechanisms and/or by differences in IgG subclasses. In the 19 transfusions with negative *in vivo* and positive *in vitro* incubations a poor recovery was found in about half of the studied platelet transfusions. This result may be explained by the removal of alloantibody coated platelets from the circulation, the temperature dependency of the antibodies to bind to platelets or false positivity of *in vitro* IgG binding assays caused by technical reasons (e.g. cut-off too low). In the 16 transfusions with a positive *in vivo* and a negative *in vitro* result only a minority of transfusions exhibited poor recoveries after 1 and 16 hours (25% and 13%, respectively). This emphasizes the fact that IgG bound to transfused platelets not necessarily leads to enhanced platelet destruction, be it alloantibodies, immune complexes or other non-specific binding of immunoglobulins to the platelets. Finally, about 40% of 142 transfusions, that showed negative results for both type of tests, demonstrated a poor platelet recovery after 1 and 16 hours.

These poor platelet recoveries may be largely caused by non-immune factors jeopardizing survival of transfused platelets.

A second reason for this discrepancy may be the difference in target antigens. No statistically significant association between the crossmatch-PIFT and the panel-PIFT was established. The discrepancy may be explained by the difference in antigenic make-up of the panel cells used in these two tests, the crossmatch-PIFT uses platelets of the 5 donors from the transfusion bag whilst the panel-PIFT uses platelets of 5 randomly selected donors, respectively. Also technical reasons may attribute to this difference such as the fact that the crossmatch-PIFT measures the fluorescence of a mixed platelet suspension of all 5 donors where as the panel-PIFT measures the fluorescence of a platelet suspension of 5 donors separately. The crossmatch-PIFT did, however, demonstrate a statistically significant association with the ELIHLA, that uses 100 random donors as a target in the ELISA technique. Nevertheless, a substantial number of discrepant results (36 of 184 transfusion) was observed. These discrepancies may be caused by difference in antigenic make-up of the target antigens used in both assays and/or a different sensitivity of the two techniques. Both tests, however, failed to show a correlation with platelet recovery.

Positive test results of all 4 tests failed to demonstrated a significant correlation with poor platelet recoveries of subsequent leukodepleted platelet transfusions after 1 hour. Only the panel-PIFT demonstrated a significant association with the recovery after 16 hours. The fact that the test results did not correlate with recovery may be caused by (i) the interference of concomitant non-immune causes of poor platelet recovery[5,33], (ii) by the tests being too frequently positive or not specific enough in detecting alloantibodies in relation to *in vivo* alloimmune platelet destruction, and/or (iii) by a low incidence of alloimmunization in our patient population receiving leukodepleted platelet transfusions. In addition, in a subgroup analysis in transfusions with a poor recovery in the absence of predefined non-immunological factors a positive crossmatch-PIFT could be demonstrated in only 12% and 7% 1 and 16 hours after transfusion, respectively. This emphasizes the fact that alloimmunization, as demonstrated by laboratory techniques, accounts for only a small amount of transfusion failures in patients receiving leukodepleted blood products[35]. The explanation that this crossmatch is insensitive, is unlikely on the basis of a substantial number of false positive tests.

We conclude that crossmatch testing is not superior to *in vitro* tests that use panel cells in relation to platelet recovery in patients receicing leukodepleted blood products. Differences between *in vitro* and *in vivo* binding of immunoglobulins to transfused platelets nor antigen composition differences between transfused and panel cells could clarify the lack of correlation of the crossmatch test with platelet recovery.

Acknowledgements

We thank Anneke Luijten and Marianne Beije for their help in collecting sera and data, Nicole Bakker for her help on performing the tests, data collection and storage, and Dew Doekharan for his help with data management.

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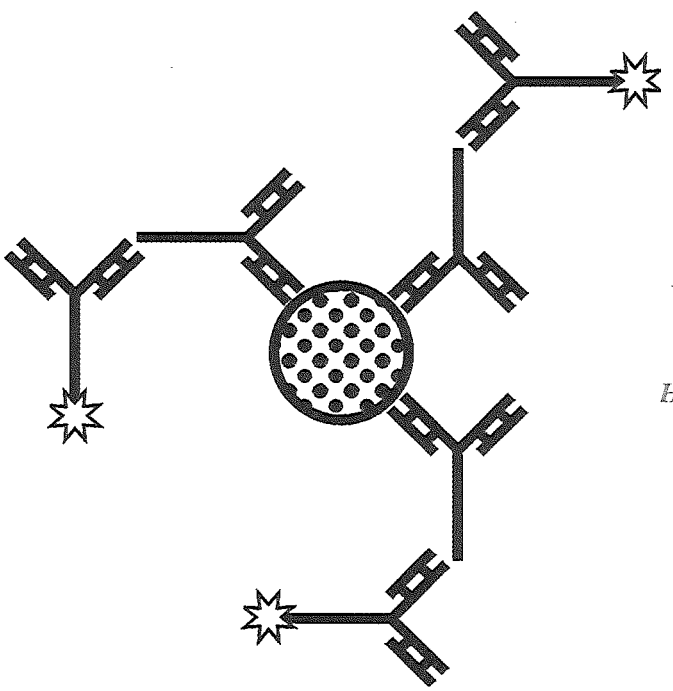
Chapter 8

The value of alloantibody detection in predicting response to HLA-matched platelet transfusions



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British Journal of Haematology 2004; 124: 244-50

Abstract

Background

Alloantibody tests demonstrate immunological causes of insufficient increments in random platelet transfusions. The value of a positive or negative test result in predicting outcome of HLA-matched transfusions in patients refractory to leukodepleted random platelet transfusions has not been assessed.

Methods and materials

We retrospectively evaluated outcome of first HLA-matched platelet transfusions in 72 patients with haematological diseases in two ways: First the strategy according to which the patient was selected for HLA-matched platelet transfusions was analysed. The strategies were: (I) results of alloantibody tests were not available; (II) result of alloantibody test was positive; (III) inspite of a negative alloantibody test. Second the outcome of the first HLA-matched transfusion was investigated in relation to the results of alloantibody tests irrespective the strategy.

Results

No significant association was found between the decision strategy and the outcome of the first HLA-matched platelet transfusions. Positive alloantibody tests, however, predicted a better outcome of the first HLA-matched platelet transfusion ($P = 0.04$ and $P = 0.03$ after 1 and 16 hours, respectively).

Conclusion

In patients refractory to random platelet transfusions positive alloantibody tests predict a better outcome of HLA-matched platelet transfusions. Patients with negative alloantibody tests, however, may benefit from HLA-matched platelet transfusions.

Introduction

Patients with hematological illnesses and thrombocytopenia following cytotoxic therapy frequently require platelet transfusions in order to prevent or treat bleeding complications. Usually pooled platelet suspensions from random donors are used. Most of these random platelet transfusions are matched for the ABO-blood group because high titers of antibodies against ABO may enhance platelet destruction and matching for ABO-blood group is relatively easy[1-3]. Donor platelets, however, are not routinely matched for human leukocyte antigens (HLA) or human platelet antigens (HPA), because of the low prevalence of antibodies against HLA- or HPA-antigens and difficulties in finding HLA- or HPA-matched platelet suspensions[4-6]. Antibodies against HLA- and HPA-antigens can cause platelet refractoriness and febrile transfusion reactions. In order to establish whether poor platelet recovery is caused by immunological (antibodies against HLA- or HPA-antigens) or by non-immunological factors (e.g. splenomegaly, older transfused platelets, fever, drugs, bleeding) a variety of tests are used to demonstrate the presence of alloantibodies[7-32]. These tests have been demonstrated to be of use in predicting a loss of response to random donor platelet transfusions in alloimmunized patients. The value of alloantibody testing in predicting the success of an HLA-matched platelet transfusion in patients refractory to random donor platelet transfusions, however, has not been established.
In order to evaluate the importance of alloantibody tests in assigning patients to HLA-matched transfusions we conducted a retrospective study in which three decision strategies (associated with distinct clinical contexts) and the results of alloantibody tests were assessed with regard to the platelet recovery of the first HLA-matched platelet transfusion. The effect of patient variables and transfusion characteristics that may have influenced platelet recoveries were studied as well in all HLA-matched platelet transfusions the included patients received.

Methods and Materials

Patients

From January 1997 to January 2002 89 patients received 619 HLA-matched platelet transfusions at the hematological department of the Erasmus MC - University Medical Center. Seventeen patients (19%), receiving 59 HLA-matched platelet transfusions, were excluded from the analysis because of a non-hematological diagnosis in 9 (testicular carcinoma in 1, ovarian carcinoma in 3 and surgery in 5) and missing data in 8 patients. Finally 560 HLA-matched platelet transfusions (91%) in 72 patients

(81%), average 8 HLA-matched platelet transfusions per patient (range 1-36), were studied (Table 1).

Table 1. Patient characteristics

Characteristics	Category	Number
Sex	Male	22
	Female	50
Diagnosis	ALL	4
	AML	29
	CLL	1
	CML	4
	MM	8
	NHL	2
	MDS	10
	AA	9
	ITP	3
	Other	2
Treatment	Chemo-/other therapy	49
	Auto SCT	5
	Allo SCT	18

ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphoblastic leukemia; CML = chronic myeloid leukemia; MM = multiple myeloma; NHL = non-Hodgkin's lymphoma; MDS = myelodysplastic syndromes; AA = aplastic anemia; ITP = idiopathic thrombocytopenic purpura; Auto SCT = autologous stem cell transplantation; Allo SCT = allogeneic stem cell transplantation.

Decision strategies

Patients, demonstrating a platelet recovery (defined below) after 1 hour of less than 20% in at least two consecutive platelet transfusions (refractory) and in whom an immune mediated cause was presumed (non-immunological causes of platelet refractoriness were absent, patients developed chills or fever following random platelet transfusions), were selected for HLA-matched platelet transfusions. Three clinical contexts that led to the decision of HLA-matched platelet transfusions were distinguished: (I) transfusion without alloantibody test result (LIFT or MAIPA result became known after the HLA-matched platelet transfusion); (II) transfusion with information on a positive alloantibody test result; (III) transfusion inspite of a negative alloantibody test result (an empiric HLA-matched platelet transfusion was given). Outcome of these three transfusion strategies were compared retrospectively.

Platelet concentrates

Platelet concentrates were obtained from the Sanquin Blood Bank South West Region and were prepared as an apheresis product from a single HLA-matched volunteer donor consisting of a wide range of platelets per transfusion ($82 - 1173 \times 10^9$). The HLA-match was classified as *compatible*, when donor and recipient shared all HLA-A and B antigens or when the donor expressed less HLA-A and B antigens than the recipient. It was designated as *crossreacting*, when cross-reactive HLA-mismatches between the donor and recipient HLA-A and B antigens were present[2]. The platelet concentrates were filtered through a PLX-5 Asahi filter (Baxter, La Chatre, France) to deplete the suspension from leucocytes. The average leukocyte number was 0.05×10^6 per platelet concentrate (range $0.00\text{-}0.20 \times 10^6$). In patients receiving a hematopoietic stem cell transplantation the platelet concentrates were irradiated with 25 Gy in order to prevent graft versus host disease (GvHD) by transfused lymphocytes[33]. The platelet concentrates were stored at 22 ^{0}C under continuous agitation for a maximum of five days. Also erythrocyte concentrates were always leukocyte depleted and irradiated for the same reasons as stated above.

Platelet transfusion results

$$\% \text{ recovery} = \frac{\text{Platelet increment } (10^9/\text{l}) \times \text{blood volume (l)}}{\text{Number of transfused platelets } (10^{11})} \times 100$$

A successful platelet transfusion was defined as a recovery of platelets of 20% or more after 1 hour or 10% or more after 16 hours after transfusion[34,35].

Tests

Assays for HLA-antibodies

I. Indirect Lymphocyte Immunofluorescence Test (LIFT)

A slightly modified method was used according to the method described elsewhere[11,19,28]. In short four parameter flow cytometry was performed on a lymphocyte population from a panel of 5 donors after incubation with patient's serum and a diluted mixture of fluorescence-conjugated goat anti-immunoglobulin G (IgG). The donor panel was simultaneously incubated with a positive and negative control serum in order to prevent inter assay variation. The patient was considered to be sensitized against a donor if the fluorescence showed more than 36% of all lymphocytes exceeding a standard threshold (determined by more than 100 negative controls). A patient was considered immunized against a broad range of HLA-antigens

(alloimmunized) if 40% or more of all 5 donors showed a positive result. The LIFT measures mainly antibodies against HLA-antigens.

II. Monoclonal Antibody-specific Immobilization of Platelet Antigens (MAIPA)
This test was performed according to Kiefel with slight modifications[14]. In short, platelet rich plasma (PRP) was stored and frozen after fixation with paraformaldehyde[10] from a pool of 100 donors. After defrosting PRP the platelets were solubilized with TBS-Nonidet P40 (BDH, Poole, Dorset, England) after successive incubation with patient's serum and a monoclonal mouse anti-human antibody against the MHC-class I molecule (Dako, Glostrup, Denmark). The solubilized platelets were added to the wells of a microtiter plate that was covered with a polyclonal rabbit anti-mouse immunoglobulin (Dako). After incubation and washing the microplates a horse radish peroxidase conjugated-rabbit anti-human antibody against immunoglobulin G was added. Again after incubating and washing ABTS-peroxide (Roche, Basel, Switzerland) was added and the extinction was measured. The extinction of a negative panel serum was subtracted from the extinction of patient's serum. When this subtraction exceeded 150 (two times the standard deviation, as was previously determined by more than 20 negative donors) the patient was considered immunized against a broad range of HLA-antigens (alloimmunized). This test detects antibodies against HLA-antigens.

Indirect Platelet Immunofluorescence Test (PIFT)
This method was used with slight modifications from description elswhere in two different techniques[10, 19]. In short, four parameter flow cytometry was performed on a panel platelet population after incubation with patient's serum and fluorescence-conjugated goat anti-IgG. The panel platelet population was derived from fresh whole blood of 5 donors separately[19] or from platelets (stored and frozen as PRP) after fixation with paraformaldehyde[10] of a pool of 100 donors. In the first technique a donor was considered to be positive when the fluoresence showed more than 17% of all platelets exceeding a standard threshold (established in more than 100 negative controls). A patient was considered immunized against a broad range of HLA-antigens if 40% or more of all donors showed a positive result. In the second technique a patient was considered immunized against a broad range of HLA-antigens if more than 20% of all platelets exceeded a specific threshold (previously determined on 20 negative controls). The PIFT measures all causes of IgG bound to platelets (antibodies against HLA- and platelet-specific antigens, autoantibodies and possibly immune complexes).

Patient variables and transfusion characteristics

The recovery after 1 and 16 hours of all HLA-matched platelet transfusions the study group received were compared to patient variables sex, diagnosis and therapy (described in Table 1) and transfusion characteristics ABO-match and HLA-match.

Statistical methods.

The association between strategies and recovery after the first transfusion or the test results and recovery after the first transfusion was investigated using Fisher's exact test in case of discrete variables, and the Kruskal-Wallis test in case of continuous outcomes. Univariate logistic regression with adjustment for patients with multiple transfusions was used to evaluate the association between the patient and transfusion characteristics and the recovery in all transfusions. All P-values are two-sided and a significance level $\alpha = 0.05$ was used.

Results

Alloantibody results of the study group

Test results in 72 patients were as follows: in 13 patients PIFT and HLA-tests (LIFT or MAIPA) were negative, in 51 patients PIFT and HLA-tests were positive. In 5 patients the PIFT was positive and the HLA-tests were negative, in 3 patients the PIFT was negative and the HLA-tests were positive.

Response to HLA-matched platelet transfusions according to strategy

Of the 72 patients 17 received HLA-matched transfusions following strategy I (decision for transfusion without information of an alloantibody test result), 39 following strategy II (decision based on a positive alloantibody test) and 15 following strategy III (decision inspite of a negative alloantibody test) (Table 2). In one patient it was not clear whether the decision was based upon a positive test result. This patient has been excluded from this analysis, but is included in the analysis on test results irrespective the strategy. The average recoveries of the first HLA-matched platelet transfusion did not differ significantly between the three strategies. The average recoveries (± standard deviation) of the transfusions after 1 hour were 42% (± 18%), 47% (± 26%) and 35% (± 27%) for strategies I, II and III (P = 0.17) and the average recoveries after 16 hours were 20% (± 20%), 32% (± 25%) and 17% (± 14%) (P = 0.10), respectively (Table 2). Successful transfusions for strategies I, II and III were noted in 94%, 85% and 73% after 1 hour (P = 0.27) and 76%, 77% and 67% after 16 hours (P = 0.71), respectively.

Table 2. Decision strategy vs. platelet recovery

Item	Strategy			P-value
	n = 17	*n* = 39	*n* = 15	
	I	II	III	
Positive HLA-test	14	39	0	-
$Recovery_{1hour}$	42% ± 18%	47% ± 26%	35% ± 27%	0.17
$Recovery_{16hours}$	20% ± 20%	32% ± 25%	17% ± 14%	0.10
$Successful\ transfusions_{1hour}$	16 (94%)	33 (85%)	11 (73%)	0.27
$Successful\ transfusions_{16hours}$	13 (76%)	30 (77%)	10 (67%)	0.71

The number of patients with a positive HLA-test (LIFT or MAIPA), the recovery (average ± standard deviation) after 1 and 16 hours and the number (percentage) of successful transfusions after 1 (recovery ≥ 20%) and 16 hours (recovery ≥ 10%) of the first HLA-matched transfusion compared to strategy I (test not available), II (positive test) and III (negative test). One patient is not included in this tabel because the decision strategy was not clear.

Response to HLA-matched platelet transfusions according to test results

Of the 72 patients HLA-tests had been positive in 54 patients (14 strategy I, 39 strategy II and 1 not clear). In 18 patients (3 strategy I and 15 strategy III) no HLA-alloantibodies could be detected in these techniques (LIFT and MAIPA). The average platelet recovery of the first HLA-matched platelet transfusion in patients with a positive HLA-test after 1 hour was 47% (± 24%) and in patients with a negative test 35% (± 25%) (P = 0.04). The average platelet recovery after 16 hours of the first HLA-matched platelet transfusion in patients with a positive test was 31% (± 24%) and 15% (± 14%) in patients with a negative test (P = 0.03) (Table 3). Successful transfusions for patients with a positive and negative test were present in 87% and 78% after 1 hour (P = 0.45) and in 80% and 61% after 16 hours (P = 0.13), respectively.

Table 3. HLA-test results vs. platelet recovery

Item	HLA-test		P-value
	n = 54	*n* = 18	
	Positive	**Negative**	
$Recovery_{1hour}$	47% ± 24%	35% ± 25%	0.04
$Recovery_{16hours}$	31% ± 24%	15% ± 14%	0.03
Successful $transfusions_{1hour}$	47 (87%)	14 (78%)	0.45
Successful $transfusions_{16hours}$	43 (80%)	11 (61%)	0.13

The recovery (average ± standard deviation) after 1 and 16 hours and the number (percentage) of successful transfusions after 1 (recovery ≥ 20%) and 16 hours (recovery ≥ 10%) compared to a positive or negative HLA-test (LIFT or MAIPA).

In this series a positive PIFT was found in 56 patients and a negative PIFT in 16 patients. The average platelet recovery after 1 hour of the first HLA-matched platelet transfusions in patients with a positive PIFT was 46% (± 25%) and in patients with a negative test 38% (± 24%) (P = 0.14). After 16 hours the average platelet recovery of the first HLA-matched platelet transfusion in patients with a positive PIFT was 30% (± 24%) compared to 17% (± 14%) in patients with a negative test (P = 0.08). Successful transfusions for patients with a positive and negative PIFT were present in 86% and 81% of cases after 1 hour (P = 0.70) and in 79% and 75% of cases after 16 hours (P = 0.21), respectively.
In the 3 patients with a positive HLA-test and a negative PIFT a successful increment was established in 100% of first HLA-matched transfusions both after 1 and 16 hours. Among the 5 patients in whom a negative HLA-test and a positive PIFT was found 80% of first HLA-matched transfusions resulted in a successful recoveries at 1 and 16 hours after transfusion.

Analysis of patient and transfusion factors

With regard to the recovery values after 1 and 16 hours of the 560 HLA-matched transfusions no differences were noted between male and female patients. There was no apparent impact of diagnosis or treatment (chemotherapy compared to autologous or allogeneic stem cell transplantation) on the values of platelet recovery either. ABO-blood group incompatibility did not predict platelet recovery, although a trend towards lower recovery 16 hours after transfusion (P = 0.07) was evident (83% of successful outcomes in ABO compatible HLA-matched transfusions compared with 69% in ABO incompatible HLA-matched transfusions, in 7 transfusions the ABO-match was not known). In 145 transfusions with a cross-reactive HLA-mismatch 77% of successful outcomes were established after 1 hour, which was significantly less (P = 0.04) than the 88% of successful outcomes in 404 HLA-compatible transfusions. No

difference in recoveries after 16 hours between cross-reactive and compatible transfusions was found, 82% of successful transfusions in compatible transfusions and 77% in incompatible transfusions (P = 0.44). In 11 of the 560 transfusions no HLA match could be found retrospectively.

Discussion

Alloantibody measurement in the serum of patients not responding to random donor platelet transfusions has become common practice. Several studies have demonstrated that alloantibody tests predict for insufficient increment of random non-leucodepleted platelet transfusions[4–6]. We and others could not find a correlation between test result and platelet recovery of random transfusions[36–38]. The importance of these tests in predicting the success of HLA-matched platelet transfusions, in patients refractory to leucodepleted random donor platelet transfusions, has not been studied.

In the retrospective study reported here 3 decision strategies (not based on an available alloantibody test result; based on a positive alloantibody test; and inspite of a negative alloantibody test) of selecting patients for HLA-matched transfusions were compared. We could not demonstrate a difference between these 3 strategies with regard to the increment of the first HLA-matched platelet transfusion. A trend, however, towards a lower average transfusion result was observed for strategy III. We may have been unable to demonstrate a significant difference between these 3 strategies due to the limited number of patients accrued during the 5 year period. In addition, a supprisingly 82% of the 17 transfusions following strategy I demonstrated a positive HLA-test in retrospect, and thus patients of both strategy-groups I and II showed serological evidence of alloimmunisation in majority.

In the second part of the analysis the results of a positive or negative HLA-test was related to the recovery of the first HLA-matched transfusion. Significantly better average recoveries of the first HLA-matched platelet transfusion were observed in the patient group with a positive HLA-test. This underscores the importance of patient selection with the help of an HLA-test for HLA-matched transfusions in a population receiving leucodepleted blood products. However, 7 of 54 patients with a positive HLA-test showed a poor recovery of the first HLA-matched transfusion suggesting a possible role of non-immune factors. These factors had not been specified or recognised by the treating physician as the cause of refractoriness, and therefore their nature remains unclear. On the other hand it is of note that 14 of 18 patients who demonstrated a negative HLA-test showed positive response to HLA-matched transfusions. The adequate recovery of HLA-matched platelet transfusions in the

absence of documented HLA sensitization cannot easily be explained but it is possible that transient non-immunological factors have played a role in the lack of response of these patients to the random platelet transfusions earlier. In addition, it is conceivable that the restricted sensitivity of HLA-tests in detecting alloantibodies is too restricted in a proportion of patients. Finally in vivo absorption of alloantibodies to transfused platelets at the time of serum sampling for the alloantibody test might have interfered.
The results of HLA-tests and PIFT demonstrated a considerable overlap. Therefore the added value of PIFT in the series as a predictor of immunization was limited. The PIFT did not demonstrate a significant correlation between test result and the average recovery of the first HLA-matched platelet transfusion, although a trend towards poorer recoveries in patients with a negative PIFT was noted. The 11% (8/72) of discrepant results between HLA-tests and PIFT may be explained by the fact that HLA-tests only measure alloantibodies against HLA-antigens and the PIFT detects also other alloantibodies (platelet-specific, autoantibodies and possibly immune complexes). In addition HLA-antigen expression on platelets is less than that on lymphocytes, which are used as targets in the LIFT. This may explain why the HLA-tests demonstrated a stronger association with increment of HLA-matched platelet transfusions than the PIFT. Furthermore, the incidence of platelet specific antibodies with or without the presence of HLA antibodies is in our and others experience low[39]. Platelet specific antibodies, therefore, are not expected to influence the outcome of this analysis.
In looking for other factors interfering with platelet recovery we considered the total 560 HLA-matched transfusions in the study group. No differences in platelet recoveries were found in relation to sex, diagnosis or therapy. We were not able to demonstrate a significant effect of ABO-incompatibility upon platelet recovery of HLA-matched transfusions. Although the study was not assigned to primarily address this issue, this is in contrast to other reports on random platelet transfusions[1–3,40]. Our patients received random ABO-matched platelet transfusions prior to HLA-matched transfusions. In this way a boost to ABO-antigens may have been avoided. We demonstrated a significantly better recovery after 1 hour of a compatible HLA-match in contrast to a cross-reactive HLA-match. This emphasizes the importance of the effort that has to be undertaken to find the best match for a patient immunized against a broad range of HLA-antigens.
In conclusion a positive HLA-test predicts for better overall recovery of HLA-matched platelet transfusions in patients refractory to random donor platelet transfusions. Patients refractory to random donor platelet transfusions with a negative HLA-test and without non-immunological factors present, however, may benefit from HLA-matched transfusions in a significant proportion of cases, although the average recovery is

considerably lower. In these patients or in patients in whom no results of HLA-tests are available an empiric HLA-matched platelet transfusion may be considered.

Acknowledgements

We thank Ferdinand de Winter and Anneke Luijten for their help in collecting the data.

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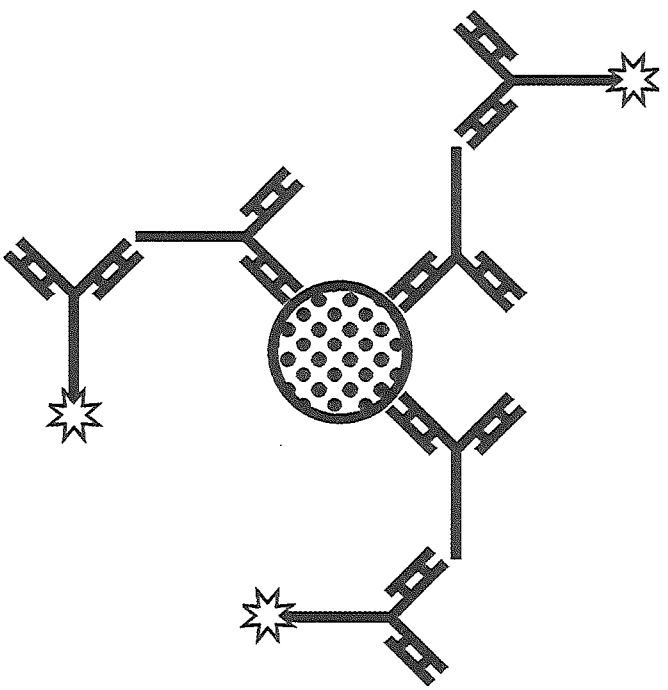
Chapter 9

General summary



Introduction

Platelet transfusions from voluntary donors are indispensable for preventing bleeding in patients who are thrombocytopenic due to their hematologic disorder (e.g. acute leukemia) or to marrow suppressive treatment (radiation, chemotherapy). A considerable proportion of platelet transfusions, however, fail to produce a good recovery after transfusion. One of the causes of enhanced donor platelet destruction results from antibody formation by the recipient against antigens present on transfused platelets. The frequency of alloantibody mediated destruction of donor platelets has been considerably diminished since the application of leukodepletion to all blood products, which is a standard procedure in many countries including the Netherlands.
In this thesis we describe and compare different tests to detect alloantibodies to platelets. We study the correlation of the results of these tests and the platelet transfusion outcome, and estimate the frequencies of alloantibody formation (in serum) as well as non-immunological factors that have been implicated in reduced survival of transfused platelets. These studies were conducted in patients who recieve leukodepleted blood products. The results of these studies lead to a reappraisal of the clinical value of platelet antibody detection.

Alloantibody detecting tests based on *in vitro* incubation

The lymphocyte, platelet and granulocyte immunofluorescence test (LIFT, PIFT and GIFT, respectively) using a cell panel from 5 random donors and a simultaneous four-channel flowcytometric technique in order to detect IgG and IgM antibodies bound to platelets, lymphocytes and neutrophils have been described in **chapter 2**. The usefulness of the LIFT and the PIFT was illustrated by the description of a patient who developed IgM and IgG alloantibodies against platelets and lymphocytes. Repeated platelet transfusion failures were noted in the presence of these alloantibodies reacting in the LIFT and PIFT (most probably alloantibodies against a broad range of HLA-class I antigens). The alloantibodies could be circumvented with the use of HLA-matched single donor platelet transfusions in this patient. All the patients who showed alloantibodies of the IgM-class also demonstrated alloantibodies of the IgG-class. The technique of simultaneous four-channel detection of IgG antibodies bound to platelets, lymphocytes and neutrophils by flowcytometry was shown to be more sensitive than the lymphocyte cytotoxic test (LCT). We standardised test results by introducing a defined threshold above which a test was regarded as positive. The threshold was established by measuring the fluorescence of a number of negative sera.

In **chapter 3** we studied the correlation between the results of the LCT, LIFT and PIFT, all using donor panel cells, in 192 prospectively and randomly selected serum samples. The routine measurement of the GIFT, described in chapter 2, was discontinued because of the large inter-assay variation, the significant background of autofluorescence from the neutrophils and its minimal clinical added value (the prevalence of antibodies against neutrophils is very low and is rarely related to platelet refractoriness). The results of the LIFT, PIFT and LCT correlated significantly with each other ($P < 0.005$). The LIFT, however, more frequently produced positive test results in relation to the PIFT and the LCT. Each of the 3 tests were found occasionally to assess alloantibodies against lymphocytes or platelets that might have been missed by any of the other two tests. In this chapter the results of LCT, PIFT and LIFT were also compared to those of a commercially available ELISA technique using a broad range of immobilized HLA-class I antigens (ELIHLA). The results of the LIFT and ELIHLA demonstrated a highly significant correlation ($\chi^2 = 63.7$; $P < 0.001$). In addition, the results of the ELIHLA and the PIFT or LCT significantly correlated as well ($\chi^2 = 42.3$; $P < 0.001$ and
$\chi^2 = 8.6$; $P = 0.003$, respectively). But the ELIHLA more frequently produced positive test results as compared to the PIFT and the LCT.

Detection of *in vivo* bound antibodies to platelets

In **chapter 4** we have described a newly developed test, the *in vivo* binding of immunoglobulins in the platelet immunofluorescence test (IVBI-PIFT). This technique compares the fluorescence histogram of IgG bound *in vivo* to platelets before a platelet transfusion with the fluorescence histogram after the platelet transfusion. We described 4 distinct patterns of fluorescence in the combined histograms (before and after transfusion). Pattern 1 (no shift of the fluorescence histogram both before and after platelet transfusion) was interpreted as no alloimmunization or complete elimination of transfused platelets from the circulation within 1 hour. Pattern 2 (enhanced fluorescence of platelets before and after transfusion as compared to that of the platelets in the transfusion bag) was assumed to represent aspecific binding of immunoglobulins to platelets (e.g. due to immune complexes and autoantibodies). Pattern 3 (greater fluorescence before platelet transfusion) was interpreted as aspecific binding of immunoglobulins or alloantibodies against formerly transfused platelets (e.g. HLA-antibodies or platelet specific alloantibodies against formerly transfused platelets). Finally, pattern 4 (increased fluorescence after platelet transfusion) was assumed to reflect the condition of alloantibodies against the transfused platelets (e.g.

HLA-antibodies or platelet specific alloantibodies). These patterns were related to the results of the indirect PIFT, which measures IgG-binding to panel platelets *in vitro*. Positive results of the indirect PIFT were more frequently associated with transfusions that demonstrated pattern 4 (enhanced binding of IgG to platelets after transfusion) in relation to pattern 1 (no enhanced binding of IgG to platelets after transfusion), P = 0.001. Poor platelet recoveries were more frequently seen in association with pattern 4 as compared to pattern 1 (P = 0.002). As expected, patterns 2 and 3 did not relate with results of the indirect PIFT nor with transfusion response *in vivo*.
The results of the IVBI-PIFT had been scored visually by a single blinded observer in the previous study. In order to eliminate interobserver variability of the visual scoring of the histograms, according to the 4 different patterns in the IVBI-PIFT, we developed a histogram subtraction method in 201 random serum samples (**chapter 5**). A histogram was created by subtracting the number of platelets per fluorescence channel (256 channels in toto) of the histogram before transfusion from those of the histogram after transfusion. Finally, the net histogram was constructed solely of the channels with a positive number of platelets of the remaining histogram. This histogram was characterized by the average number of remaining platelets in duplicate tests, Events Post Subtraction (EPS), and the average mean value of the curve of the remaining platelets in duplicate, Mean Channel Post Subtraction (MCPS). The interobserver disagreement rate in the IVBI-PIFT, defined as less than 3 of 5 blinded observers with identical visual scoring, was 13% (26/201) in our study. In the remaining 175 serum samples in which 3 or more blinded observers agreed on the score, named the consensus visual score, the pattern-score was compared to the results of the histogram subtraction. Pattern 4 appeared distinguishable from patterns 1 and 2 with EPS ($P < 0.0001$). Pattern 4 could be distinguished from pattern 3 with the MCPS ($P < 0.0001$). The combination of EPS (≥ 1100) and MCPS (≥ 30) reliably predicted pattern 4 in relation to patterns 1, 2 and 3 ($P < 0.001$). However, the platelet transfusions associated with pattern 4 by the consensus visual scoring method nor the histogram subtraction method predicted for platelet recovery at 1 and 16 hours following transfusion.

Predictive value of alloantibody detecting tests

The relative importance of immunological and non-immunological factors in relation to the survival of transfused platelets in randomly selected patients was examined in **chapter 6**. We prospectively studied the results of 4 alloantibody tests (ELIHLA, LCT, LIFT and PIFT) and their relation with the platelet recoveries in 181 transfusions

from 97 patients. Various established non-immunological factors that might have influenced the survival of transfused platelets were taken into account. None of the 4 tests appeared to correlate with platelet recoveries at 1 or 16 hours after transfusion. Only the combination of ELIHLA and PIFT or that of LIFT and PIFT demonstrated a statistically significant association with platelet recoveries at 16 hours ($P = 0.04$ and $P = 0.03$, respectively). The non-immunological factors under consideration were: diagnosis, therapy, splenomegaly, fever (>38.2 ^{0}C), disseminated intravascular coagulation, graft versus host disease, use of Amphotericin B and storage time of platelet transfusions from acquisition to donation. Significantly reduced platelet recoveries were apparent only in relation to splenomegaly ($P < 0.01$ at 1 hour and $P = 0.02$ at 16 hours after transfusion) and in relation to platelet transfusions that had been stored for more than 3 days ($P = 0.01$ at 1 hour and $P = 0.03$ at 16 hours after transfusion).

In **chapter 7** we set out to further explore the lack of a relation between alloantibody tests and platelet transfusion outcome. Since we regarded the crossmatch test as the "golden standard" with regard to alloantibody testing (transfused platelets incubated with patient's serum in the PIFT technique, named crossmatch-PIFT) we related the results of this assay with the transfusion outcome. We did not find a positive relationship between a positive crossmatch-PIFT and poor platelet recoveries at 1 and 16 hours after a random donor platelet transfusion. A subgroup analysis in platelet transfusions in absence of the non-immunological factors that might have influenced platelet transfusion results (splenomegaly and storage time of platelets of more than 3 days) revealed a positive crossmatch-PIFT in 12% of transfusions with a poor outcome (based on recovery estimate) at 1 hour and in 7% of transfusions with a poor response at 16 hours. The results of the crossmatch-PIFT (*in vitro* incubation of patient's serum with transfused platelets) correlated with those of the IVBI-PIFT (*in vivo* binding of alloantibodies to transfused platelets, which serves as an *in vivo* correlate of the crossmatch), $P = 0.02$. In addition, the results of the crossmatch-PIFT (using transfused platelets as a target) were compared to those of 2 screening tests using donor panel antigens as a target (panel-PIFT and ELIHLA). The results of the crossmatch-PIFT and the panel-PIFT did not correlate ($P = 0.25$). This was in contrast to a significant positive relationship between the results of the crossmatch-PIFT and the ELIHLA ($P = 0.02$). None of the two alloantibody tests using donor panel cells as a target predicted platelet recoveries at 1 and 16 hours after transfusion correctly.

The predictive value of alloantibody tests with regard to outcome of HLA-matched platelet transfusions was studied in a selected patient population, refractory to random platelet transfusions and with no apparent involvement of non-immunological factors (**chapter 8**). The platelet recoveries at 1 and 16 hours after the first HLA-matched

platelet transfusions in 72 patients were analysed according to the clinical decision by which the patient had been assigned to HLA-matched platelet transfusions. The decisions were taken in the following context: (I) results of alloantibody tests were not (yet) available when the patient was assigned to HLA-matched platelet transfusions; (II) the alloantibody test had been positive; and (III) the alloantibody test had been negative. No significant relationship was found between the context in which the decision for HLA-matched platelet transfusions was made and the outcome of the transfusion. However, in patients selected for HLA-matched transfusions according to strategy III there was a trend towards a poorer recovery at 1 and 16 hours after transfusion. The recoveries of the first HLA-matched transfusion were also investigated in relation to the results of alloantibody tests irrespective of the strategy. The latter comparison demonstrated that a positive alloantibody test predicted a better average response to the first HLA-matched platelet transfusion (assessed at 1 hour, $P = 0.04$, and 16 hours after transfusion, $P = 0.03$). In order to evaluate other influences on the efficacy of HLA-matched platelet transfusions, we studied the effect of a variety of patient and transfusion factors in relation to the response to 560 HLA-matched transfusions in the 72 patients. No differences in platelet transfusion response were observed for gender, diagnosis or therapy (chemotherapy compared to autologous or allogeneic stem cell transplantation). ABO-blood group incompatibility tended towards lower recoveries at 16 hours after transfusion, $P = 0.07$ (83% of successful outcomes in ABO compatible HLA-matched transfusions compared with 69% in ABO incompatible HLA-matched transfusions), but not at 1 hour. A *cross-reactive* (the donor expresses HLA-class I antigens that are not present in the acceptor) HLA-match demonstrated a significantly lower recovery after 1 hour, $P = 0.04$, than a *compatible* (all HLA-class I antigens of the donor are present in the acceptor) HLA-match; no difference was observed after 16 hours.

Conclusions of the studies of this thesis

Chapter 2

In this chapter we describe a double colour flowcytometric technique in which simultaneously alloantibodies from the IgG- and IgM-class can be detected. In the studied patient population IgM alloantibodies were not found in the absence of alloantibodies from the IgG-class.

Chapter 3

The results of 4 different screening tests for alloimmunization based on different serological principles (LCT, PIFT, LIFT and ELIHLA) are significantly correlated. The LIFT and ELIHLA are more frequently positive than the LCT and PIFT, thus being more sensitive for the detection of HLA-antibodies.

Chapter 4

A method of a modified PIFT in which the *in vivo* binding of IgG preferentially to the transfused platelets can be measured (named IVBI-PIFT) is described. The results of this method are correlated with those of the *in vitro* binding of IgG in the indirect PIFT and with the outcome of the studied platelet transfusions.

Chapter 5

A histogram subtraction method is described to objectivate the visual score of the IVBI-PIFT as used in chapter 4.

Chapter 6

The different alloantibody assays, which showed a good intercorrelation with respect to serological results (chapter 2), did turn out to be predictive for platelet transfusion outcome when used as screening tests in an unselected population in need for platelet transfusions. Reasons for the absence of this correlation are given and non-immunological factors that jeopardize platelet survival were identified.

Chapter 7

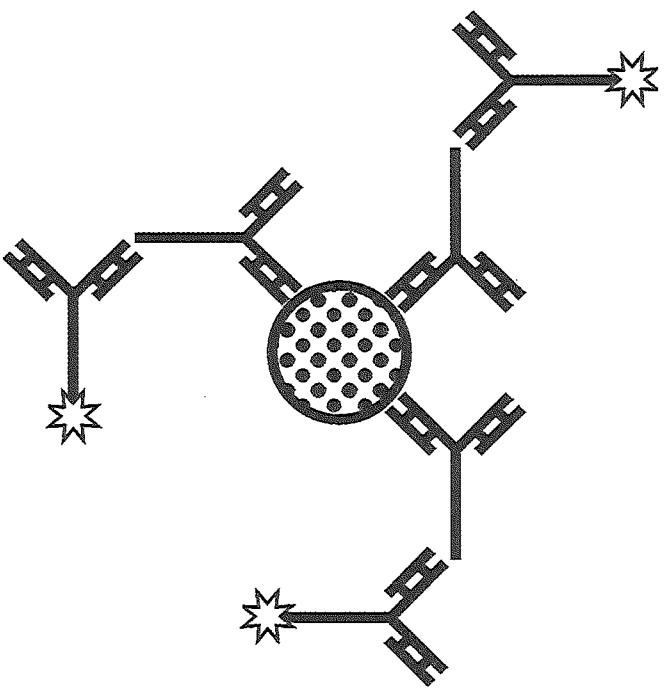
The results of a platelet crossmatch test in the PIFT technique are significantly related to those of the IVBI-PIFT and ELIHLA, but not to those of the indirect PIFT. The results of these 4 tests (crossmatch-PIFT, IVBI-PIFT, ELIHLA and indirect PIFT), however, do not predict the outcome of a subsequent platelet transfusion in a non-selected patient population.

Chapter 8

HLA-matched platelet transfusions in patients who are refractory to random donor platelet transfusions and in whom non-immunological factors for enhanced platelet destruction are excluded have a significantly higher average recovery if the patients have a positive alloantibody test in relation to those with a negative test result.

Chapter 10

Algemene samenvatting



Inleiding

Trombocytopenie is frequent aanwezig bij patiënten met hematologische ziekten en wordt veroorzaakt door: (i) de hematologische ziekte (verdringing van het gezonde beenmerg door de maligne aandoening of een primaire aplasie), (ii) de therapie (chemotherapie, immunotherapie, bestraling, omgekeerde afstoting of transplantaatfalen na een beenmerg of perifere stamceltransplantatie), (iii) het toegenomen verbruik van trombocyten (diffuse intravasale stolling, actief bloedverlies of autoantistoffen), of (iv) een abnormale distributie van trombocyten (splenomegalie). Indien het trombocytenaantal lager is dan 10 x 10^9 per liter worden profylactisch trombocytentransfusies toegediendom bloedingen te voorkomen[1,2]. Deze transfusies bestaan uit een mengsuspensie van de trombocyten van 5 willekeurige donoren.
Vierentwintig tot 44% van de profylactische trombocytentransfusies zijn niet succesvol als gevolg van immunologische en/of niet-immunologische factoren[3 - 6]. Indien immunologische of niet-immunologische oorzaken van een lage trombocytenopbrengst persisteren, is de patiënt *refractair* geworden voor trombocytentransfusies van 5 willekeurige donoren. De immunologisch veroorzaakte refractaire status berust op de vorming van antistoffen tegen klasse I humane leukocyten antigenen (HLA-klasse I), antistoffen tegen trombocytspecifieke antigenen, op autoantistoffen of op circulerende immuncomplexen[7,8]. Het risico op alloimmunisatie tegen HLA-klasse I antigenen neemt toe naar gelang de hoeveelheid leukocyten per transfusieproduct. Een andere risicofactor voor alloimmunisatie is een eerder doorgemaakte zwangerschap[6,9,10]. Onder de niet-immunologische oorzaken van een refractaire status worden vele klinische factoren geschaard die de overleving van getransfundeerde trombocyten negatief beïnvloeden. Voorbeelden van niet-immunologische oorzaken van een refractaire status zijn splenomegalie, medicatie, koorts, diffuse intravasale stolling en actief bloedverlies[3].
Verschillende testen, die antistoffen tegen HLA-antigenen aantonen, worden toegepast om alloimmunisatie als oorzaak van een refractaire status te onderscheiden van de aanwezigheid van niet-immunologische factoren. In dit proefschrift is onderzoek gedaan naar de waarde van verschillende testen die alloantistoffen tegen HLA-antigenen bepalen. De waarde van deze testen is afgemeten aan de overeenkomst met referentietesten. Ook is de voorspellende waarde van testuitslagen voor het resultaat van trombocytentransfusies bestudeerd. De verschillende studies zijn verricht bij patiënten die alleen gefiltreerde bloedprodukten hebben ontvangen. Het gevolg van de leukodepletie van bloedprodukten is de reductie van alloimmunisatie als oorzaak van de refractaire status voor trombocytentransfusies[9,11-21].

Onderlinge vergelijking van verschillende alloantistoftesten

In **hoofdstuk 2** wordt een flowcytometrische techniek beschreven waarmee tegelijkertijd IgG en IgM antistoffen tegen trombocyten, lymfocyten en granulocyten kan worden vastgesteld, de PIFT, LIFT en GIFT, respectievelijk. Een afkappunt voor positiviteit van de PIFT, LIFT en GIFT (afzonderlijk IgG en IgM) werd bepaald met behulp van een panel van 10 niet geïmmuniseerde donoren. Op deze manier kunnen de resultaten van deze testen worden geobjectiveerd. Tevens wordt een patiënt beschreven met een refractaire status voor ongetypeerde trombocytentransfusies. Bij deze patiënt werden IgG en IgM antistoffen aangetoond in de PIFT en LIFT (antistoffen tegen HLA-klasse I antigenen). De refractaire status voor trombocytentransfusies bleek te kunnen worden doorbroken door HLA-overeenkomstige trombocytentransfusies toe te dienen. In alle bestudeerde patiënten die alloantistoffen van de IgM-klasse lieten zien, werden ook alloantistoffen van de IgG-klasse gevonden.
De gevoeligheid en betrouwbaarheid van een commercieel verkrijgbare ELISA-techniek met een breed scala van HLA-antigenen (ELIHLA) wordt vastgesteld in **hoofdstuk 3**. Hierin worden de resultaten van de ELIHLA vergeleken met de uitslagen van de LCT, PIFT en LIFT. Een statistisch significante correlatie wordt gevonden tussen de uitslagen van alle 4 de testen. De LIFT en de ELIHLA zijn het best met elkaar gecorreleerd. Tevens lijken deze 2 testen het meest frequent positief te zijn als uiting van alloimmunisatie tegen HLA-klasse I antigenen.
Aangezien het verband tussen de resultaten van verschillende *in vitro* testen en de trombocytenopbrengst *in vivo* frequent niet overeenkomen, wordt in **hoofdstuk 4** een door ons ontwikkelde techniek beschreven. In deze techniek, de IVBI-PIFT genoemd, wordt de *in vivo* binding van IgG aan getransfundeerde trombocyten vastgesteld. Het fluorescentie histogram van de trombocyten na transfusie geïncubeerd met een anti-IgG-antistof (verbonden met een fluorochroom) wordt vergeleken met hetzelfde histogram voorafgaande aan de trombocytentransfusie. Hierbij worden de trombocyten uit de transfusiezak als negatieve controle gehanteerd. Er werden 4 verschillende patronen onderscheiden: patroon 1 laat geen toegenomen fluorescentie van de trombocyten zien (geen immunisatie of volledige destructie van getransfundeerde trombocyten binnen het uur); patroon 2 laat een toegenomen fluorescentie van de trombocyten voor en na transfusie zien in vergelijking met de trombocyten uit de transfusiezak (aspecifieke binding van antistoffen aan trombocyten, bijv. circulerende immuuncomplexen); patroon 3 laat een toegenomen fluorescentie voorafgaande aan de transfusie zien (antistoffen tegen voorgaande transfusies); en patroon 4 toegenomen fluorescentie na transfusie (geïnterpreteerd als alloimmunisatie). Het patroon 4,

toegenomen fluorescentie na de trombocytentransfusie, werd geïnterpreteerd als een positieve testuitslag van de IVBI-PIFT. In dit hoofdstuk wordt aangetoond dat patroon 4 (alloimmunisatie) vaker met een positieve indirekte PIFT overeenkomt (P = 0.001) dan patroon 1 (geen alloantistoffen tegen trombocyten). Tevens wordt bij trombocytentransfusies met patroon 4 vaker een slechte trombocytenopbrengst aangetroffen dan bij transfusies met patroon 1, 67% (16/24) versus 31% (22/72) van de transfusies, respectievelijk (P = 0.002).
Na introductie van de IVBI-PIFT in onze kliniek bleek de visuele patroonscore onderhevig te zijn aan inter-individuele variatie tussen verschillende observatoren. In **hoofdstuk 5** wordt een methode beschreven, waarbij de visuele patroonscore vervangen wordt door een geobjectieveerde uitslag van de IVBI-PIFT, de zogenaamde histogram-subtractie methode. In dit hoofdstuk bleek de visueel gescoorde patronen in 26 van de 201 (13%) trombocytentransfusies niet overeen te komen tussen 5 observatoren. De techniek van histogram-subtractie in de IVBI-PIFT is als volgt: het aantal trombocyten per fluorescentiekanaal (256 fluorescentiekanalen per histogram) voor en na de trombocytentransfusie worden van elkaar afgetrokken. Een nieuw histogram wordt gemaakt van de resterende trombocyten van die fluorescentiekanalen met meer dan of evenveel trombocyten na transfusie als voor transfusie, de overige fluorescentiekanalen worden op 0 trombocyten gedefiniëerd. Uiteindelijk wordt van dit resulterende histogram het gemiddeld aantal trombocyten (EPS) en het gemiddelde fluorescentie kanaal (MCPS) van de duplo-meting gegenereerd. Het blijkt dat EPS goed differentiëerde tussen patroon 4 en patroon 1 en 2, terwijl MCPS goed differentiëert tussen patroon 4 en patroon 3. De combinatie van EPS $\geq$ 1100 en MCPS $\geq$ 30 onderscheidt het best tussen patroon 4 en de overige patronen (P < 0.001 voor het verschil). De visuele score en de histogram subtractie is echter niet gecorreleerd met de trombocytenopbrengst 1 en 16 uur na transfusie. Dit komt waarschijnlijk door de aanwezigheid van eerder genoemde niet-immunologische factoren en de lage prevalentie van immunologische factoren.

Voorspellende waarde van alloantistoftesten

Het belang van immunologische en niet-immunologische factoren als verklaring voor een slechte trombocytenopbrengst wordt beschreven in **hoofdstuk 6**. In dit hoofdstuk wordt aangetoond dat er geen verband tussen de resultaten van 4 verschillende antistofmetende testen (LCT, ELIHLA, PIFT en LIFT) en een slechte trombocyten opbrengst na 1 en 16 uur in onze populatie kon worden gevonden. Alleen een combinatie van een positieve ELIHLA en PIFT of een combinatie van een positieve

LIFT en PIFT lieten een relatie zien met een slechte trombocytenopbrengst 16 uur na een trombocytentransfusie, P = 0,04 en P = 0,03 respectievelijk. Deze combinatie van positieve testen was aanwezig in slechts 2% van de trombocytentransfusies. Van de niet-immunologische factoren was splenomegalie gecorreleerd met een slechte trombocytenopbrengst na 1 en 16 uur (P = 0,05 en P = 0,02, respectievelijk) evenals een bewaartijd van de trombocytentransfusies van meer dan 3 dagen (P = 0,01 en P = 0,03, respectievelijk). Opvallend was dat een significant slechtere trombocytenopbrengst werd gezien bij transfusies van patiënten met chronische myeloïde leukemie (CML), allogene stamceltransplantatie (alloSCT) en van het mannelijk geslacht. Deze correlatie werd met name veroorzaakt door de aanwezigheid van splenomegalie als interfererende factor en kon in een multivariate analyse niet worden bevestigd. Geconcludeerd wordt in dit hoofdstuk dat de alloantistoftesten niet betrouwbaar de trombocytenopbrengst kunnen voorspellen in een niet-geselecteerde patiëntenpopulatie.

Om te onderzoeken waarom de correlatie tussen alloantistofmetende testen en trombocytenopbrengst slecht is, wordt in **hoofdstuk 7** als "gouden standaard" een trombocytenkruisproef vergeleken met de uitkomst van verschillende testen en de trombocytenopbrengst. De trombocytenkruisproef bestaat uit een *in vitro* incubatie van het serum van de patiënt met de getransfundeerde trombocyten, waarbij eventuele binding wordt gemeten in de PIFT-techniek. In dit hoofdstuk wordt beschreven dat er ook geen correlatie bestaat tussen de trombocytenkruisproef en de trombocytenopbrengst. Van de transfusies met een slechte transfusieopbrengst in de afwezigheid van eerder gedefiniëerde klinische factoren (splenomegalie en een trombocytenbewaartijd van meer dan 3 dagen) blijkt slechts 12% na 1 uur en 7% na 16 uur een positieve trombocytenkruisproef te hebben. De teleurstellende correlatie tussen trombocytenkruisproef en trombocytenopbrengst wordt verder onderzocht door het verband te bestuderen: tussen (i) *in vitro* en *in vivo* binding van alloantistoffen aan getransfundeerde trombocyten en tussen (ii) trombocytenkruisproef en screeningstesten die een ander donoren panel hanteren. De *in vitro* en *in vivo* binding van IgG aan getransfundeerde trombocyten (trombocytenkruisproef versus IVBI-PIFT) zijn statistisch geassocieerd (P = 0,02). Er wordt echter geen relatie gevonden tussen de kruisproef en de panel-PIFT, P = 0,25. Wel wordt een associatie tussen de resultaten van de kruisproef en de ELIHLA aangetoond (P = 0.02). De resultaten van de kruisproef, de IVBI-PIFT, de panel-PIFT noch de ELIHLA laten een verband met de trombocytenopbrengst zien. Hieruit wordt geconcludeerd dat het niet zinvol is sera te screenen op de aanwezigheid van alloantistoffen bij alle patiënten die een trombocytentransfusie krijgen.

De relevantie van het toepassen van alloantistoftesten op een geselecteerde patiëntenpopulatie, die refractair zijn voor ongetypeerde trombocytentransfusies,

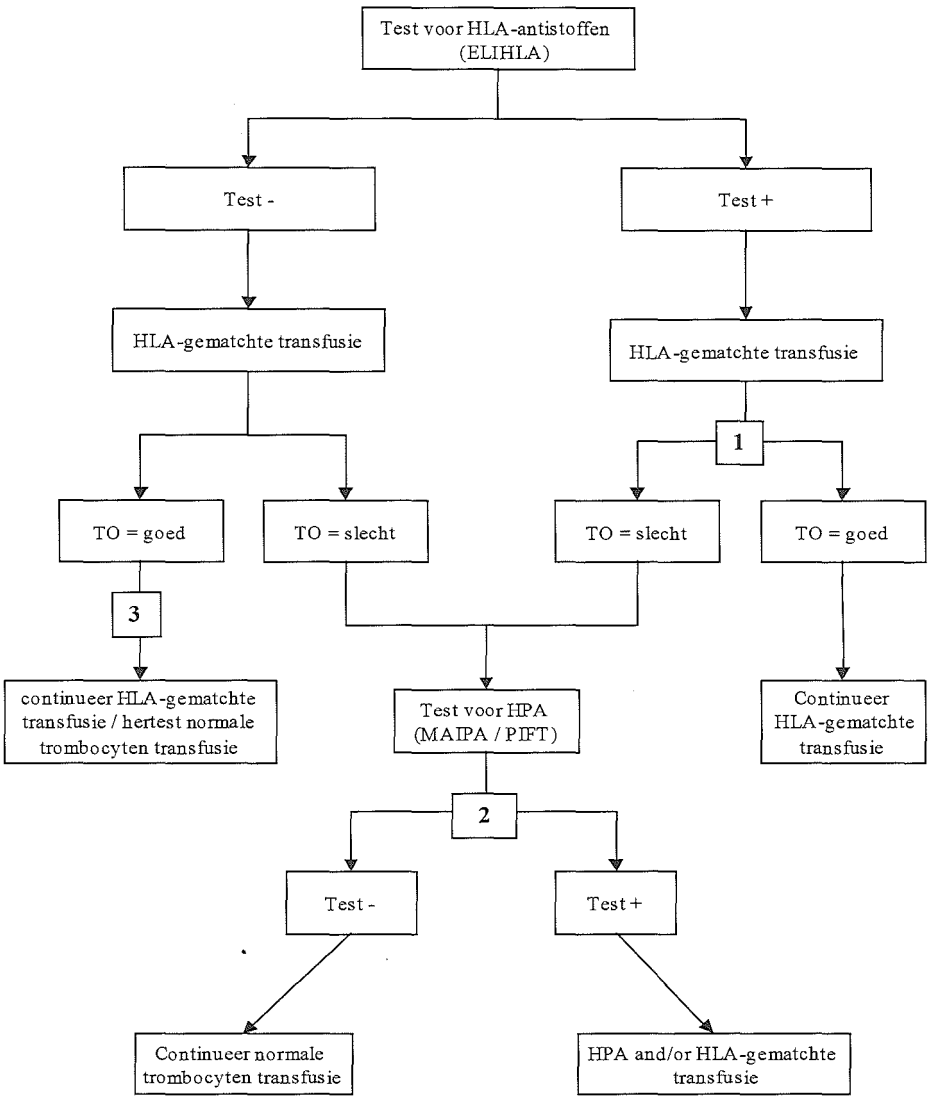
wordt beschreven in **hoofdstuk 8**. In dit hoofdstuk wordt van alle patiënten, die in het ErasmusMC van januari 1997 tot januari 2002 een HLA-overeenkomstige trombocytentransfusie ontvingen, de trombocytenopbrengst van de eerste HLA-overeenkomstige trombocytentransfusie vergeleken met de resultaten van alloantistoftesten. In eerste instantie worden 3 omstandigheden betreffende het (beschikbare) resultaat van een alloantistof test, alvorens werd besloten tot een HLA-overeenkomstige transfusie, in verband gebracht met de trombocytenopbrengst van deze eerste HLA-overeenkomstige transfusie. Deze omstandigheden waren: (I) geen resultaat van een alloantistofmetende test was beschikbaar, (II) een positieve alloantistofmetende test was bekend en (III) een negatief resultaat van een alloantistofmetende test was bekend. Er werd geen significant verschil gevonden tussen de trombocytenopbrengst van de eerste HLA-overeenkomstige transfusie en de omstandigheid waarin de van de beslissing voor deze transfusie werd genomen. Wel werd een tendens tot slechtere trombocytenopbrengst opgemerkt bij strategie III. In tweede instantie werden de resultaten van alloantistoftesten vergeleken met de trombocytenopbrengst onafhankelijk van de beslissingsstrategie. In deze vergelijking bleek een positieve alloantistoftest significant gecorreleerd met een betere trombocytenopbrengst 1 en 16 uur na de trombocytentransfusie, P = 0,04 en P = 0,03, respectievelijk. Opvallend was dat toch nog 78% en 61% van patiënten met een negatief testresultaat een goede transfusie opbrengst hadden 1 en 16 uur na de HLA-overeenkomstige transfusie. Kortom patiënten, die refractair zijn voor ongetypeerde trombocytentransfusies in afwezigheid van niet-immunologische factoren en die een positieve alloantistoftest hebben, laten een betere trombocytenopbrengst van HLA-overeenkomstige transfusies zien dan patiënten met een negatieve test. Maar een aanzienlijk deel van de patiënten, die refractair zijn voor ongetypeerde trombocytentransfusies, met een negatieve alloantistoftest kunnen wel degelijk baat hebben bij een HLA-overeenkomstige transfusie.

In deze patiëntengroep werden van alle 560 HLA-overeenkomstige transfusies ook nog vastgesteld dat *compatibele* HLA-overeenkomstige transfusies (patiënt heeft alle HLA-klasse I antigenen die de donor heeft) een betere trombocytenopbrengst hadden na 1 uur in vergelijking met een *kruisreagerende* transfusie (patiënt heeft niet alle HLA-klasse I antigenen die de donor heeft), P = 0.04. Dit benadrukt het belang van een goed HLA-overeenkomstigheid tussen donor en ontvanger. Hiernaast werd een trend tot een slechtere trombocytenopbrengst na 16 uur bij ABO-incompatibele HLA-overeenkomstige transfusies waargenomen, P = 0.07.

Conclusie

Er zijn vele technieken beschikbaar, die alloantistoffen tegen trombocyten detecteren. In studies, waarbij de patiënten geen leukocytenarme bloedprodukten kregen is aangetoond dat de trombocytenopbrengst samenhangt met het testresultaat. Aangezien de waarde van de verschillende testen niet duidelijk was in patiënten die gefiltreerde bloedprodukten ontvangen, hebben wij verschillende testen in deze patiëntengroep onderzocht. Wij vonden dat, ook al kwamen de resultaten van de meeste testen goed met elkaar overeen, de voorspellende waarde van alle onderzochte testen ten opzichte van de trombocytenopbrengst zeer beperkt is. Het lijkt daarom niet zinvol om alloantistof testen uit te voeren bij alle patiënten die gefiltreerde bloedprodukten ontvangen. Wel lijkt het zinvol de testen te selecteren voor patiënten die refractair zijn en bij wie geen niet-immunologische factoren aanwezig zijn die de verkorte overlevingsduur van trombocyten kunnen verklaren. Omdat bij de vergelijking van de verschillende testen onderling geen van de serologische testen superieur bleek te zijn, adviseren wij de test te hanteren, die de minste vals positieve resultaten geeft en die het meest eenvoudig is om uit te voeren. In onze studies was dat de ELIHLA.
Indien patiënten refractair voor trombocytentransfusies zijn en indien er geen immunologische oorzaken aanwezig zijn die de trombocytenopbrengst beïnvloeden bevelen wij aan een proeftransfusie met HLA-overeenkomstige trombocyten toe te dienen en een alloantistofmetende test uit te voeren. De HLA-overeenkomstige trombocytentransfusie heeft dan 94% en 76% (hoofdstuk 8) kans op een goede transfusie opbrengst 1 en 16 uur na de transfusie. Mocht de test negatief zijn dan kan bij een volgende transfusie het geven van ongetypeerde trombocyten opnieuw worden overwogen. Bij een positieve test mag men aannemen dat alloimmunisatie de oorzaak is van het slechte transfusieresultaat van ongetypeerde trombocyten, tenzij ook HLA-overeenkomstige transfusies geen goede opbrengsten geven. Men kan in het laatste geval de aanwezigheid van plaatjesspecifieke antistoffen overwegen die niet met de ELIHLA gemeten kunnen worden. Deze situatie is in de praktijk echter zeer zeldzaam. Een voorstel voor de transfusie-strategie indien is besloten tot het toedienen van een HLA-overeenkomstige trombocytentransfusie is weergegeven figuur 1. Enkele belangrijke beslispunten zijn verder toegelicht in de legenda.

Figuur 1. Voorgesteld diagram voor het vervolg na een HLA-overeenkomstige trombocytentransfusie



ELIHLA = ELISA met een panel van 100 willekeurige donoren (Quikscreen™), TO = transfusie opbrengst, HLA = humane leukocyten antigenen, HPA = humane plaatjesspecifieke antigenen, MAIPA = monoclonal antibody-specific immobilization of platelet antigens assay, PIFT = plaatjes immunofluorescentie test.

Verschillende beslispunten zijn hieronder toegelicht:

1. *Patiënten die geen trombocytenopbrengst van ongetypeerde transfusies hebben in de afwezigheid van niet-klinische factoren en met een positieve alloantistoftest moeten voortaan HLA-overeenkomstige transfusies krijgen. Volgens ons onderzoek hebben de*

HLA-overeenkomstige trombocytentransfusies dan een kans van 87% en 80% na 1 en 16 uur op een goede trombocytenopbrengst. Als deze patiënten bij herhaling geen goede opbrengst hebben op een HLA-overeenkomstige transfusie dient een test uitgevoerd te worden om de aanwezigheid van humane (bloed)plaatjesspecifieke antistoffen (HPA-antistoffen) te onderzoeken.

2. *Bij patiënten die een slechte trombocytenopbrengst van ongetypeerde transfusies hebben in de afwezigheid van niet-klinische factoren en die bij herhaling geen opbrengst hebben van HLA-overeenkomstige transfusies dient de aanwezigheid van antistoffen tegen HPA-antigenen te worden onderzocht. Indien er antistoffen tegen HPA-antigenen aanwezig zijn, dienen HLA- en HPA-overeenkomstig transfusies te worden toegediend. Indien er geen antistoffen tegen HPA-antigenen aantoonbaar zijn, is het zinloos verdere HLA-overeenkomstig trombocytentransfusies toe te dienen. Er kunnen dan weer ongetypeerde trombocytentransfusies worden gegeven, eventueel in een hogere dosis.*
3. *Voor patiënten, die geen trombocytenopbrengst na ongetypeerde transfusie hebben in de (schijnbare) afwezigheid van niet-klinische factoren met een negatieve alloantistoftest en een goede opbrengst van een HLA-overeenkomstige transfusies, wordt geadviseerd eenmalig een herhaalde toediening van een ongetypeerde trombocytentransfusie te verrichten. Op deze manier kunnen passagere niet-immunologische oorzaken, welke eerder niet herkend zijn, worden uitgesloten. Als deze ongetypeerde trombocytentransfusies vervolgens een goede trombocytenopbrengst heeft, kan de patiënt weer ongetypeerde trombocytentransfusies ontvangen. Indien de ongetypeerde transfusie een slechte opbrengst toont, dient de patiënt in het vervolg HLA-overeenkomstige transfusies te ontvangen.*

De verschillende onderzoeken in dit proefschrift leiden tot de volgende conclusies:

1. Verschillende alloantistofmetende testen laten een goede overeenkomstigheid in de resultaten zien. Wij raden daarom aan om de meest eenvoudige test te gebruiken, welke in onze onderzoeken de ELIHLA was.

2. Het regelmatig testen van de sera van ongeselecteerde patiënten, die gefiltreerde bloedprodukten ontvangen, op de aanwezigheid van alloantistoffen is niet zinvol, aangezien de uitslagen niet de trombocytenopbrengst voorspellen.

3. Dit geldt niet zonder meer voor het uitvoeren van deze testen bij een patiëntengroep die refractair is voor ongetypeerde trombocytentransfusies in de afwezigheid van niet-immunologische factoren. De gemiddelde opbrengst van HLA-overeenkomstige trombocytentransfusies is binnen deze populatie beter bij patiënten met een positieve alloantistoftest. Een goed transfusieresultaat bij patiënten met een negatieve alloantistoftest is echter zeker niet uitgesloten.

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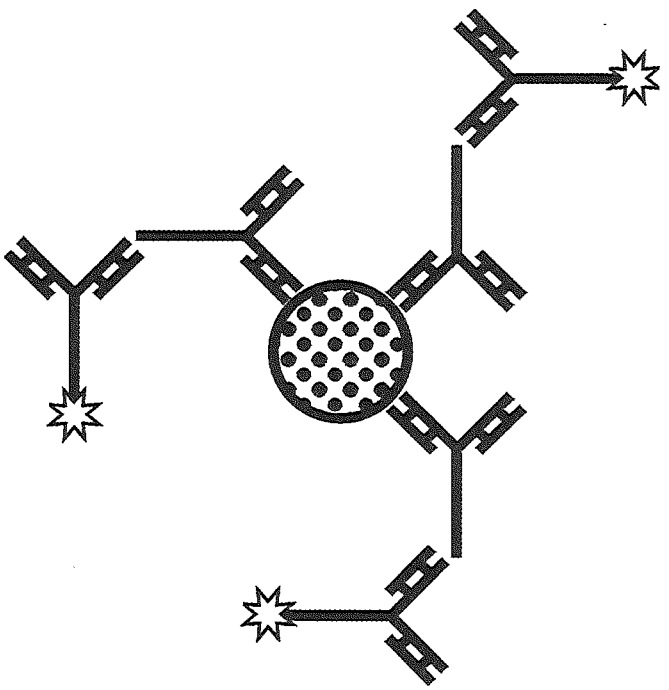
Chapter 11

General discussion



The problematic place of alloantibody tests in clinical practice

In 2003 an international forum has addressed the value and applicability of alloantibody tests in general[1]. The forum emphasized the large differences that exist between countries in their policy of alloantibody detection (used test, frequency of alloantibody detection and when to applicate HLA-matched transfusions) (Tabel 1).

Table 1. The value of alloantibody tests according to an international forum

Country	Preferred alloantibody test	The predictive value of alloantibody tests (in relation to platelet refractoriness)	Standard leukodepletion
France	ELIHLA	Good	Yes
Denmark	LCT	Possible	No
Poland	LCT and LIFT	Not clear	No
Austria	MAIPA	Possible	Yes
Japan	LCT	Good	Yes
USA	ELIHLA	Not clear	No
Finland	FlowPRA	Not clear	No
Spain	LCT	Good	Yes
Germany	LCT and ELISA	Possible	Yes
Netherlands	ELIHLA	Not clear	Yes
England	LCT and ELISA	Not clear	Yes

ELIHLA = Enzyme linked immunosorbent human leukocyte antigen assay (Quikscreen™), ELISA = Enzyme-linked immunosorbent assay, flowPRA = Platelet radioactive antiglobulin detection by flowcytometry with latex beads with HLA-class I antigens, LCT = Lymphocyte cytotoxic test, LIFT = Lymphocyte immunofluorescence test, MAIPA = Monoclonal antibody-specific immobilization of platelet antigens assay

Secondly, the presumed predictive value of alloantibody tests as interpreted by experts from these 11 countries was widely variable. Three experts reported a good correlation between alloantibody test results and platelet recovery, 3 experts reported a possible correlation, and 5 experts could not provide evidence for a correlation. Apparently there is a lot of disagreement with regard to the utility of these tests in clinical practice.

Alloantibody testing: the questions

In order to clarify the obscurity of this issue we studied the place of alloantibody tests in clinical practice in this thesis. We focus on two aspects of alloantibody testing against platelets: technical aspects and clinical value. Technical aspects concerned

comparison of techniques based on three different principles: complement lysis (LCT), immunofluorescence (PIFT and LIFT) and the enzyme linked immunosorbent assay (ELISA). The LCT, the oldest and best validated technique, is used in many laboratories worldwide. Newer techniques like immunofluorescence read by flowcytometry, and ELISA have the advantage of greater sensitivity and more objective reading of the results. We studied the correlation of the results of different screening tests and modified the techniques in certain studies with the intention of mimicing the *in vivo* situation. Thus we wished to assess whether any of the tests would be superior in measuring alloantibodies against platelets. In order to assess the clinical value of alloantibody testing against platelets we correlated the test results with the *in vivo* recovery of platelets after transfusion.

Alloantibody tests

Serological considerations on platelet alloantibody tests

Overall we found a good correlation between the results of the various alloantibody tests. Discrepancies in the results in individual cases were noted. We assume that these discrepancies might have been caused by: (i) differences in the specificity of the alloantibodies that are detected, (ii) differences in the target antigens in the panel (qualitatively an quantitatively), and (iii) differences in the test conditions.

Ad (i) the specificity of the antibody. Antibodies specific exclusively for HLA-antigens were detected in the LIFT and an ELISA using HLA-class I antigens from 100 donors as a target (ELIHLA). Antibodies against HLA and platelet specific antigens were detected in the PIFT-technique and its modifications (IVBI-PIFT and crossmatch-PIFT). In the techniques that use immunofluorescence also aspecifically bound IgG, e.g. immune complexes, can be found. The role of this aspecifically bound IgG under test circumstances is uncertain, but seems to be of minor importance. One of the reasons that we did not detect large discrepancies between the tests is that in alloimmunization by platelet transfusions the antibodies are most frequently directed against HLA-antigens or against HLA and platelet specific antigens simultaneously. Rarely, these antibodies are directed against platelet specific antigens alone[2,3].

Ad (ii) antigen composition of the panel. We used fresh cells of 5 random donors as a panel in the LCT, LIFT and PIFT. It may be argued that the panel was relatively small and discrepancy in test results may be caused by restricted antigen composition of the panel. However, comparison of LCT, LIFT and PIFT with ELIHLA, in which a broad range of HLA-antigens of 100 donors is present, showed good correlations. This suggests that the distribution of the HLA-antigens on donor cells in a panel of 5 would be

representative. Lymphocytes and platelets serving as panel cells both express HLA-antigens, but platelets express HLA-antigens in a much lower density. This renders the LIFT more sensitive for detection of HLA-antibodies than the PIFT.
Ad (iii) differences in test conditions between the studied test were numerous. These variations include e.g. the use of second antibodies (complement activation, fluorescence or light-absorbance), duration of incubation, applied temperature (*in vitro* versus *in vivo*), number of washing steps, and the way of analyzing the results (automated versus visual). By comparing the results of the different tests we could not pinpoint factors that positively or negatively influenced the test results.

Comparing the results of different tests with a reference test

In spite of serological differences a highly significant correlation was found between the results of the different tests. This positive correlation between test results may also be explained by the characteristics of the study population. The tested patients had a low risk of alloimmunization due to the fact that we did not select patients at high risk of sensitization (except in chapter 8) and that patients always received leukodepleted blood products. The percentage of negative test results in this population proved to be between 85 and 90%. This imbalance in favour of negative results in each of the techniques may, therefore, have biased the correlation of the test results.
In order to exclude the influence on the herefore described imbalance in the population, we related the test results to a reference test in order to establish its reliability. This way of establishing the reliability of serologic tests in detecting alloantibodies in relation to a reference test is widely applied in literature, e.g. for the LCT[4-6], the PIFT and LIFT[7-10], the ELISA[11-14], the MAIPA[15,16], the monoclonal ^{125}I-labeled anti-IgG assay (^{125}I-MA)[17], the flowcytometry of latex beads coated with HLA class I antigens[18,19] and the commercially available ELISA tests with a broad range of HLA class I antigens[20-22]. In these studies the correlation of a test with a reference test is regarded as evidence for the reliability of the particular test, i.e. a good specificity and sensitivity in relation to a reference test. In this thesis we used the LCT, commonly used in the literature, as a reference test in chapter 2 and 3. The indirect PIFT was used in chapter 4, in order to study the impact of micro-environmental factors in relation to the IVBI-PIFT. The crossmatch-PIFT, finally, was used in chapter 7, because it is theoretically the best test in predicting alloantibody mediated platelet destruction.
The ELIHLA appears to perform best as a screening tool for alloimmunization of HLA-antigens on the basis of (i) its good correlation with the LCT, the indirect PIFT and the crossmatch-PIFT and (ii) its relatively easy applicability and time-sparing character in relation to other tests.

Clinical value

Alloantibody testing and platelet transfusion outcome

A second aspect of alloantibody testing we addressed in this thesis is the predictive value of test results for transfusion outcome. Data on this aspect are still scarcely documented (see below). Our results revealed an unsatisfactory predictive value of each of the screening tests (LCT, LIFT, PIFT, and ELIHLA). Also the two crossmatch tests (crossmatch-PIFT and IVBI-PIFT) failed to reliably predict the transfusion outcome.

This lack of prediction might have been influenced by 2 important factors: (i) the concomitent presence of non-immunological factors that jeopardize platelet survival following transfusion and (ii) the use of leukodepleted blood products. The impact of non-immunological factors upon platelet recovery *in vivo* in our patient population was clearly established for splenomegaly and a storage time of platelet transfusions of more than 3 days. Other non-immunological factors described in literature might have had a role as well. Due to their relative infrequency they did not have enough power in our study to demonstrate a correlation with platelet recovery in a univariate analysis. The second factor, the influence of filtration of blood products in order to rigorously deplete all blood products from leukocytes, is separately discussed below.

The value of alloantibody testing according to literature

The published data on the value of alloantibody tests in predicting platelet transfusion outcome is limited. In the era before the introduction of systemic leukodepletion of blood products the probability of alloimmunization was 4- to 8-fold greater than today. In those days under those transfusion conditions the LCT has been validated by Bishop with regard to the prognostic value for transfusion increment[23,24]. In a prospective study a multivariate analysis that considered both immunological and non-immunological factors in 941 *non-leukodepleted* platelet transfusions showed a positive correlation of a positive alloantibody test with poor platelet transfusion outcome. In contrast, the results of a multivariate analysis by Doughty[25] taking into account non-immunological factors did not confirm the relationship between the results of the LCT and the outcome of 266 *non-leukodepleted* platelet transfusions.

The value of alloantibody test results in relation to the outcome of *leukodepleted* platelet transfusions had previously been assessed in only one study until our studies[26]. In the latter study no association between a positive LCT and a poor outcome of 439 platelet transfusions in 42 patients was found. The study was also analysed according to multivariate approach taking into account non-immunological factors. Our study on the value of alloantibody tests in predicting response to platelet transfusions was

conducted in 181 *leukodepleted* platelet transfusions in 97 patients[27]. In our study non-immunological factors were taken into account in the multivariate analysis. No relation was found between the results of the LCT, LIFT, PIFT and ELIHLA and platelet recoveries at 1 and 16 hours after transfusion.

Five longitudinal studies on the prevalence of positive alloantibody tests in patients receiving serial *leukodepleted* platelet transfusions have been described[3,28-31]. Fabris demonstrated a positive ELISA test detecting alloantibodies against HLA-class I antigens in 4 (67%) of 6 refractory patients as compared to no alloantibodies in 19 non-refractory patients[28]. Non-immunological factors were not significantly different in the 2 groups. A positive MAIPA and/or a positive LCT was demonstrated by Kurz in 44 (54%) of 81 patients refractory to random platelet transfusions[29]. Non-immunological factors, however, were not taken into account in this study. A prospective study by Legler in 145 patients demonstrated 40 (28%) refractory patients. Of these refractory patients a positive test was found in 7 (5%) in the absence of non-immunological factors[3]. A case-control study by Alcorta demonstrated a positive LCT in 16 (31%) of 52 refractory patients in relation to 1 (2%) of 52 non-refractory patients. This difference was statistically significant in a multivariate analysis taking into account non-immunological factors[30]. A prospective study by Novotny demonstrated a positive PIFT and LCT in 31 (39%) of 79 patients refractory to random donor platelet transfusions in relation to 17 (15%) of 115 non-refractory patients without taking non-immunological factors into account[31].

Impact of leukodepletion on screening for alloantibodies

Leukodepletion of blood products has been shown to be of major importance with respect to reduction of the incidence of alloimmunization. Platelet refractoriness may be reduced from approximately 24% without leukodepletion to 3% with rigorous leukodepletion as we discussed in the introduction[32-40] (chapter 1). This reduction in the incidence of platelet refractoriness was recently confirmed by a study from Seftel. In this study the frequencies of alloimmunization and refractoriness to random platelet transfusions before and after the introduction of leukodepletion to blood products in Canada were compared[41]. A decrease in alloimmunization from 19% to 7% and in alloimmune platelet refractoriness from 14% to 4% were found.

In our studies all blood products were leukodepleted, henceforth the incidence of alloimmunization was expected to be low. For this reason an increased rate of false positive and false negative test results of alloantibody tests was to be expected in our study population.

False negativity

Because of the reduced incidence of alloimmunization following the application of leukodepleted blood products the relative contribution of non-immunological factors to platelet transfusion failures will increase. As a consequence, a higher rate of false negative results of the alloantibody tests is to be expected. In our studies the proportion of false negative test results ranged from 31 to 55% in relation to a poor recovery at 1 hour after platelet transfusion (Table 2).

Table 2. False positive and false negative rates of the different alloantibody tests

Test	False negative rate	False positive rate
LCT[1]	41%	69%
LIFT[1]	40%	60%
PIFT[1,2]	39-41%	50-53%
ELIHLA[1,2]	38-41%	50%
IVBI-PIFT-visual[3,4]	31-55%	33-43%
IVBI-PIFT-automated[2,4]	43-55%	43-65%
Crossmatch-PIFT[2]	40%	46%

The false negative and false positive rates are calculated for the platelet recovery at 1 hour after transfusion of the lymphocyte cytotoxic test (LCT), the lymphocyte and platelet immunofluorescence test (LIFT and PIFT, respectively), the enzyme linked immuno-sorbent assay with a large panel of immobilized HLA-antigens (ELIHLA), in vitro binding of immunoglobulins in the PIFT according to the visual scoring system (IVBI-PIFT-visual) or according to the automated generation of results (IVBI-PIFT-automated) and a crossmatch test according to the PIFT technique (crossmatch-PIFT).
[1] Chapter 6, [2] Chapter 7, [3] Chapter 4, [4] Chapter 5

Reduction of false negative test results can be obtained by selecting patients for these tests on the basis of the absence of non-immunological factors (patient selection).

False positivity

The percentage of false positive test results was estimated by us by combining the rate of false positive test results in the hallmark study of Bishop (sensitivity 38% and specificity 85% in the LCT)[22] and the assumed reduction in refractory patients (for detail see introduction to the thesis, chapter 1). By combining these two factors the false positive rate can be estimated according to the formula[42]:

$$\text{False positive} = \frac{(1-\text{sensitivity}) \times \text{not refractory patients}}{(1-\text{sensitivity}) \times \text{not refractory patients} + \text{specificity} \times \text{refractory patients}}$$

This results in an expected false positivity rate of 91% [0.15 x 207 / (0.15 x 207 + 0.38 x 7)] of the LCT in patients receiving leukodepleted transfusions with less than 5×10^6 leukocytes per transfusion. This 91% is a high value that is subject to significant error. Factors that influence this value are e.g. the selection bias of patients in the studies used to determine the reduction in platelet refractoriness or the number of transfusions per patient in the study of Bishop[23].

The percentage of false positive test results of the alloantibody tests in our studies ranged from 33 to 69% (Table 2). The high number of false positive tests is of major concern and compromises their usefulness as screening tests. As antibodies (IgG) bound to the surface of transfused platelets *in vivo* may not necessarily lead to their destruction and in patients not immunized by leucocytes or pregnancy a positive alloantibody titer may be low, test positivity in a random population receiving leukodepleted blood products may not have clinical consequences. Also variations in IgG-subclass composition might explain the high false positive test rate as some subclasses are more effective in the destruction than others. A reduction in false positive results can be obtained by applying the tests to a selected category of patients at high risk of sensitisation, e.g. in those showing a poor recovery after platelet transfusion.

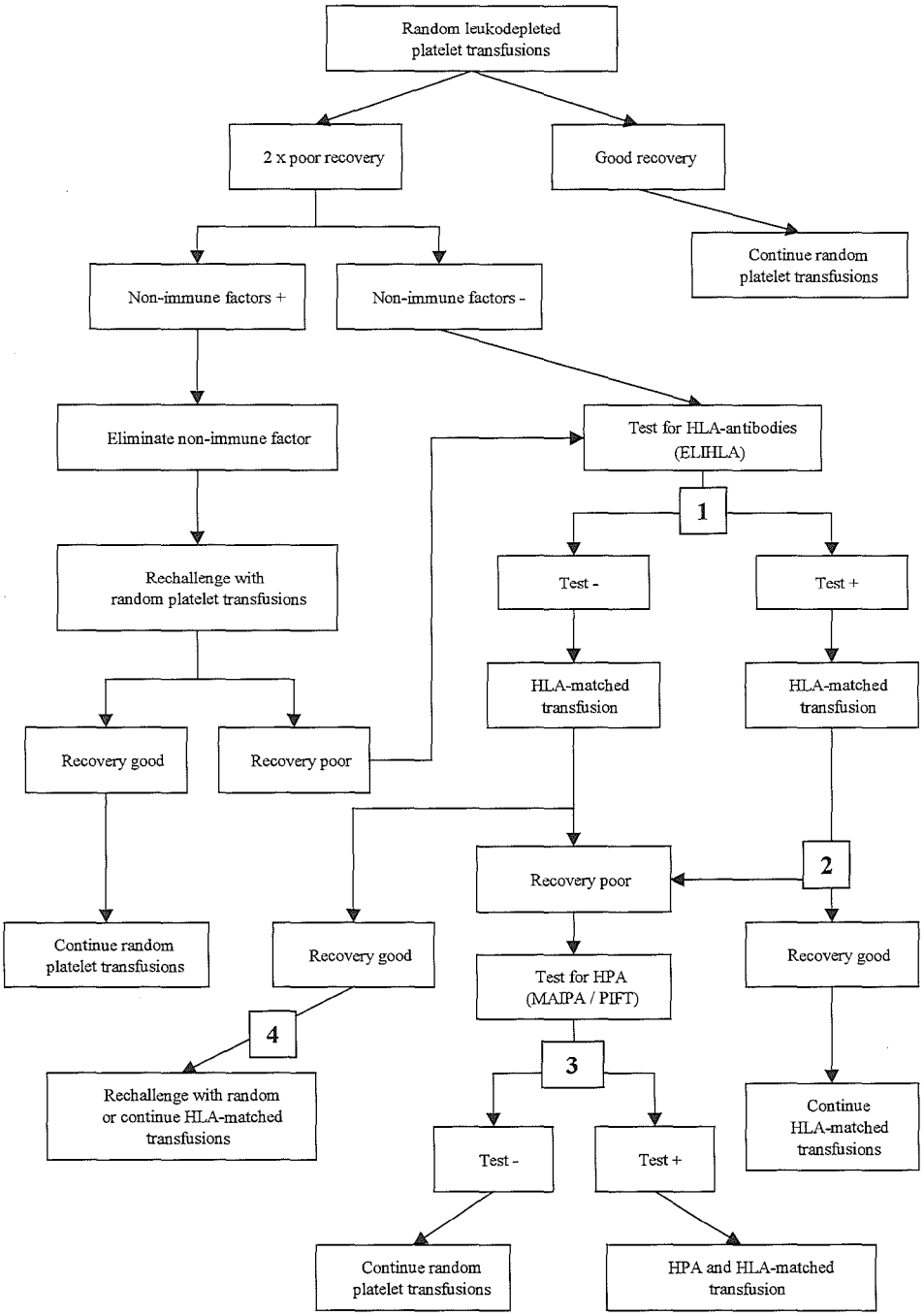
Alloantibody testing: possible answers

Alloantibody testing in clinical practice

As alloantibody tests do not qualify as a screening test in patients at low risk for alloimmunization is there any place for alloantibody testing at all? In chapter 8 we demonstrated an increased average platelet recovery after HLA-matched platelet transfusions in a selected patient population (refractory to random platelets and in the absence of non-immunological factors) who showed positive alloantibody tests. In this particular patient group characterized by an increased risk of refractoriness by alloimmunization the predictive properties of the alloantibody tests were much better. In this way the results of alloantibody tests can lend support to the decision to administer HLA-matched platelet transfusions to patients refractory to random donor platelets. In addition, the results of alloantibody tests can provide information as to whether further investigations with regard to the involvement of platelet-specific

alloantibodies are warranted. Finally, a negative alloantibody test might guide the transfusion management of a trombocytopenic patient who responded well to HLA-matched platelets leading to a rechallange with random donor platelets in order to exclude transient non-immunological factors. An algorithm for the administration of random donor and HLA-matched platelet transfusions on the basis of the results of our studies is shown in figure 1. Particular decision points in this algorithm are highlighted in the text.

Figure 1. A possible algorithm of platelet transfusion and test policy



ELIHLA = ELISA with a panel of 100 random donors (Quikscreen™), HLA = human leukocyte antigen, HPA = human platelet specific antigen, MAIPA = monoclonal antibody-specific immobilization of platelet antigens assay, PIFT = platelet immunofluorescence test.
A further enlightenment of decision points of this algoritmh are described below:

1. *Patients with a poor recovery of 2 consecutive random platelet transfusions (i) in whom non-immune factors are treated and a rechallenge with random platelet transfusion fails or (ii) in whom non-immune factors are absent, it is recommended that an alloantibody test (e.g. ELIHLA) be performed. Since the test will not sufficiently discriminate recipients with or without a response to HLA-matched platelet transfusions, the patient should be assigned to receive HLA-matched platelet transfusions irrespective the result of the alloantibody test. The expected success rate of HLA-matched platelet transfusions in these patients will be 73% and 67% at 1 and 16 hours after transfusion according to the results presented in chapter 8.*
2. *We recommend that patients refractory for random donor platelet transfusions in the absence of non-immunological factors with a positive alloantibody test continue to receive HLA-matched platelet transfusions. According to our studies the success rate of HLA-matched transfusions in these patients is 87% and 80% at 1 and 16 hours after transfusion. When these patients experience repeated failures of the HLA-matched platelet transfusions, antibodies against human platelet antigens (HPA) should be considered and efforts to elucidate the presence of concomitant alloantibodies against HPA-antigens seem appropriate.*
3. *In patients refractory for random donor platelet transfusions in the absence of non-immunological factors with repeated failures of HLA-matched platelet transfusions antibodies against human platelet antigens (HPA) should be investigated. In case antibodies against HPA-antigens can be demonstrated, it would seem reasonable to try and transfuse HPA- and HLA-matched platelet transfusions. If no antibodies against HPA-antigens can be established, further application of HLA-matched platelet transfusions may not be useful. One may consider these patients to assign to a policy of random donor platelet transfusions at higher dosages and/or in combination with immunosuppressive agents.*
4. *Patients refractory for random donor platelet transfusions (apparently) in the absence of non-immunological factors with negative alloantibody tests and successful HLA-matched platelet transfusions, may not be sensitisized to HLA-antigens. It may be worth the effort to evaluate the effect of a rechallenge with random donor platelet transfusions. In this way transient non-immunological factors, that had not been recognised, can be overcome. If this rechallenge with random donor platelet transfusion is successful the patient should be reassigned to receive random donor platelet transfusions. If the rechallenge is unsuccessful HLA-matched platelet transfusions should be administered henceforth.*

No screening at all?

Based on our studies we advocate to test for alloimmunization in selected patients only: those being refractory to random platelets and after exclusion of non-immunological factors. But even among this subgroup the risk of alloimmunization may differ considerably. Previous pregnancy has proved to be one of the strongest predictors for platelet refractoriness in different studies[3,43,44]. Also it is conceivable that patients with a rare type or mixture of HLA-class I antigens will preferentally develop alloantibodies against commonly prevalent HLA-class I antigens in the donor population. At this moment a retrospective study is conducted in which we wish to identify specific HLA-class I antigens associated with an increased risk of alloimmunization. It is also possible that certain combinations of HLA-class A, B and C antigens show a greater tendency for alloimmunization. The latter enhanced likelihood of alloimmunization might relate to the amino acid triplet containment of exposed parts of the HLA-class I molecules as recently described by Duquesnoy[45,46]. Among the large number of possible triplets only a small and particular fraction of triplets may be responsible for causing alloimmunization against donor platelets.

Previous studies have suggested a greater probability of alloimmunization in patients with HLA-DR2[47-49]. This relationship, however, might have been dependent on the number of leukocytes in the transfused blood products and could not be confirmed in a population receiving leukodepleted blood products[50]. It is also possible that the relationship of alloimmunization with HLA-DR2 does not depend on the low frequency of the HLA-antigen itself, but it might be an epiphenomenon of the fact that patients with HLA-DR2 appear high producers of immunoglobulins against different antigens (high responders), so that screening tests demonstrating alloantibodies more often turn positive.

If in future studies specific HLA-class I antigens, specific combinations of HLA-class I antigens or certain amino acid triplets of HLA-class I antigens appear to predispose for alloimmunization, these patients might profit from being monitored with alloantibody tests. This would hold particularly for female patients after pregnancies. In these selected subsets of patients HLA-matched platelet transfusions may be applied earlier in the platelet transfusion treatment on the basis of positive alloantibody tests. It might perhaps be advantages that these subsets of patients will receive HLA-matched platelets on forehand in order to prevent platelet refractoriness, but the availablility of sufficient compatible donors is likely to be small. The answers to these questions are directly relevant for optimized platelet transfusion support, in which severe bleeding complications at the time of refractoriness for random platelets may be prevented.

Final conclusions

In this thesis we investigated a series of tests that detect alloantibodies to platelets. These investigations leads us to conclude:

1. The various tests correlated statistically significantly with each other. In view of this we recommend to use the fastest and most easy applicable test (i.e. ELIHLA in our studies)

2. Screening any patients receiving leukodepleted blood products for alloimmunization is not useful as the predictive value with regard to the response to platelet transfusions is unsatisfactory.

3. An HLA-matched platelet transfusion in patients refractory to random donor platelet transfusions in the absence of non-immunological factors may have a good survival even when the alloantibody test result has been negative. The average recovery, however, of HLA-matched platelet transfusions is better in patients with a positive test.

4. In refractory patients with a negative test and a good response to HLA-matched platelet transfusions a rechallenge with random donor platelets is recommended. In refractory patients with a positive alloantibody test and a poor recovery of an HLA-matched transfusion further investigations with regard to the involvement of platelet-specific alloantibodies appear warranted.

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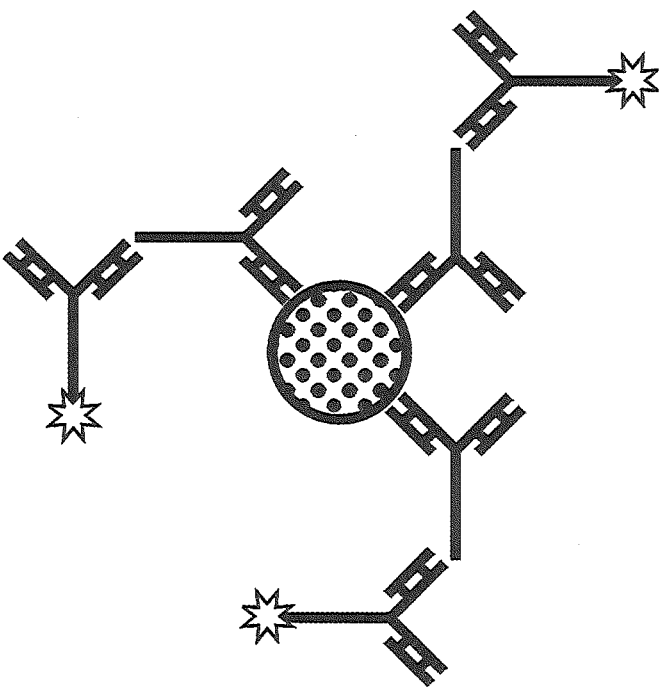
Abbreviations

AA	Aplastic anemia
AlloBMT/SCT	Allogeneic bone marrow or stem cell transplantation
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATG	Anti-thymocyte globulin
AutoBMT/SCT	Autologous bone marrow or stem cell transplantation
BDIS	Becton Dickinson
BSA	Bovine serum albumin
CCI	Corrected count increment
CI	Confidence interval
CLL	Chronic lymphoblastic leukemia
CML	Chronic myeloid leukemia
Crossmatch-PIFT	Crossmatch of patient's serum and transfused platelets in the PIFT technique
DIC	Disseminated intravascular coagulation
DLI	Donor lymphocyte infusion
D_{time}-A_{time}	Time from acquisition of platelets till donation
EDTA	Ethylenediamine tetraacetic acid
ELIHLA	Enzyme linked immunosorbent human leukocyte antigen assay (Quikscreen^{TM})
ELISA	Enzyme-linked immunosorbent assay
EPS	Events post subtraction
GAHu	Goat-anti human
HD/HL	Hodgkin's disease or Hodgkin's lymphoma
HLA	Human leukocyte antigen
HPA	Human platelet antigen
FACScan	Fluorescence activated cell scan
FITC	Fluorescein isothiocyanate
FSC	Forward light scatter characteristics
GIFT	Granulocyte immunofluorescence test
GvHD	Graft versus host disease
^{125}I-MA	Monoclonal ^{125}Iodide-labelled anti-IgG assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
In vivo-PIFT	IVBI-PIFT to which the histogram subtraction method is applied
IQR	Interquartile range

ITP	Idiopathic thrombocytopenic purpura
IVBI-PIFT	In vivo binding of immunoglobulins according to the PIFT technique
LCT	Lymphocyte cytotoxic test
LIFT	Lymphocyte immunofluorescence test
LDS	Laser dye solution
MAIPA	Monoclonal antibody-specific immobilization of platelet antigens assay
MCPS	Mean chanell post subtraction
MDS	Myelodysplastic syndromes
MM	Multiple myeloma
NHL	Non-Hodgkin's lymphoma
OR	Odds ratio
Panel-PIFT	Indirect-PIFT (PIFT technique using panel cells from 5 random donors)
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidiumiodide
P(S)IFT	Platelet (suspension) immunofluorescence test
PNPP	Phosphate p-nitrophenylphosphate
PRA(T)	Platelet radioactive antiglobulin (test)
PRP	Platelet rich plasma
RAEB-t	Refractory anemia with excess of blasts in transformation
SSC	Sideward light scatter characteristics
TO	Transfusieopbrengst

Chapter 12

Dankwoord
&
Curriculum vitae



Dankwoord

Het is vanzelfsprekend dat een promotieonderzoek tijdens een voltijdse opleiding tot internist en hematoloog alleen tot stand kan komen dankzij de hulp van vele anderen. Ik ben dan ook alle mensen die hebben bijgedragen aan dit proefschrift zeer dankbaar voor hun inspanningen en de tijd die zij hieraan besteed hebben.

Allereerst wil ik professor Löwenberg bedanken voor het feit dat hij mij in staat heeft gesteld om op zijn afdeling te promoveren. Tevens is zijn inzicht en schrijfstijl een inspiratie geweest voor verschillende delen van dit proefschrift. Zijn belangstelling gedurende vele jaren, niet alleen voor de vorderingen omtrent deze onderzoekingen maar ook voor mijn opleidingstraject op weg naar de hematologie, heb ik altijd zeer gewaardeerd.

Mars van 't Veer heeft mij al die jaren begeleid en voorzien van denkbeelden, arbeidskrachten, hardware en informatie. Zonder zijn bezieling en doorzettingsvermogen, als er onverwachte resultaten werden gevonden, had ik het zeker niet al die jaren volgehouden. Het laatste jaar moest Mars ook wel eens zuchten als zijn E-mail box overladen werd met artikelen of andere stukken uit dit proefschrift. Toch wist hij altijd tijd vrij te maken overdag, 's avonds of als het nodig was zelf 's nachts. Heel hartelijk dank voor dit alles en ik verheug mij op de vele inhoudelijke discussies die wij ongetwijfeld nog zullen hebben in de toekomst.

De meeste laboratoriumhandelingen zijn verricht door Jos de Veld op het immuno-hematologisch laboratorium in het ErasmusMC – Daniel den Hoed (voorheen de Dr. Daniel den Hoed kliniek). Jos was steeds weer bereid om protocolen in de praktijk te brengen en ook de artikelen kritisch door te lezen en van commentaar te voorzien. In het immuno-hematologisch laboratorium hebben in de loop van de jaren ook Patricia van den Broek, Petra van der Spoel en Nicole Bakker veel testen uitgevoerd en geanalyseerd.

Wim de Vries heeft mij wegwijs gemaakt in de immuno-hematologie op zijn eigen onnavolgbare manier. Zijn ideëen omtrent de verschillende technieken van alloantistoftesten zijn duidelijk herkenbaar in verschillende hoofdstukken van dit proefschrift. Tevens heeft hij kritisch gekeken naar de meerdere artikelen uit dit proefschrift.

De data analyse werd voor het grotendeels verricht door Ronnie van der Holt van de afdeling statistiek in het ErasmusMC - Daniël den Hoed. Ook heeft hij steeds met zijn welbekende kritische blik gekeken naar de artikelen, wat de kwaliteit zeker ten goede is gekomen.

Anneke Luijten en Marianne Beije hebben vele klinische data verzameld en vastgelegd in de verschillende databases gedurende meerdere jaren. Vooral het achterhalen van de trombocytentransfusiezakjes was niet altijd eenvoudig, maar vele zakjes zijn toch uiteindelijk getest dankzij hun inspanningen. Ferdinand de Winter en Dew Doekharan hebben voor de verschillende onderzoeken databases gemaakt, wat de dataverzameling en verwerking op een hoog niveau heeft gebracht. Karola van Rooyen heeft meerdere figuren in diverse artikelen bewerkte en opgemaakt met vaak grote spoed en enthousiasme. Hans Kneefel heeft geholpen met de vormgeving en figuren van het uiteindelijke boekje.
Jan Willem Gratama heeft, nadat onze eerste samenwerking lag in het verwerken van data van mijn afstudeerdonderzoek in een artikel, meegeschreven aan verschillende artikelen. Dat laatste heeft met zekerheid geleid tot een betere leesbaarheid van deze artikelen.
Reinier van der Linden heeft de flowcytometer onderhouden en deed de kwaliteitscontroles van de flowcytometer. Reinder Bolhuis heeft personeel en materiaal ter beschikking gesteld op zijn laboratorium.
Kees Sintnicolaas als grondlegger van het immunohematologisch laboratorium en onderzoek naar alloantistoffen tegen trombocyten in het ErasmusMC – Daniel den Hoed (voorheen de Dr. Daniël den Hoed kliniek) heeft frequent meegedacht en meegeschreven aan verschillende artikelen.
Mies Kappers-Klunne en Huub van Vliet van de afdeling hematologie in het ErasmusMC – centrumlocatie hebben mij veel geleerd over trombocyten en antistoffen, tevens hebben zij meegschreven aan een van de artikelen van dit proefschrift.
Mijn oud-collegae in het Medisch Centrum Rijnmond Zuid locatie Zuider (voorheen Zuider Ziekenhuis) ben ik dankbaar voor de "waarneem-uurtjes" met betrekking tot mijn patiëntenzorg. Deze vervanging heeft mij in staat gesteld om tussen de bedrijven door via "de tunnel" naar het immuno-hematologisch laboratorium in "De Daniel" te gaan, als daar aanleiding voor was.
Mijn collegae in het ErasmusMC tijdens en na mijn opleiding tot hematoloog ben ik dankbaar voor de extra "schrijftijd" die ik heb gekregen in de laatste maanden om dit proefschrift af te kunnen ronden.
Mijn vrienden, de "Snoekjes" en mijn schoonfamilie waren altijd zeer belangstellend omtrent de voortgang van dit proefschrift en altijd bereid mee te denken of, indien nodig, de helpende hand toe te steken.
Mijn paranimfen Karen & Kees ben ik dankbaar voor hun gezelligheid, steun en ideëen bij de voorbereiding van deze promotie. Ik ben blij dat jullie op deze dag aan mijn zijde staan.

Mijn ouders zijn altijd een voorbeeld geweest voor het feit dat als je hard werkt het mogelijk is om vele verschillende activiteiten succesvol te combineren. Mijn moeder had altijd tijd voor een bemoedigend gesprek, een plakje cake en een kopje koffie in moeilijke tijden. Tijdens de werkzaamheden voor dit proefschrift en mijn opleiding tot internist en hematoloog ben ik er achter gekomen, dat ik stilzwijgend de kritische houding ten opzichte van vele factoren ongemerkt van mijn vader heb overgenomen. Dat is, denk ik, een van de belangrijkste drijfveren voor mijn interesse in wetenschappelijk onderzoek.

Manne en Jakob, jullie glimlach, knuffel of de aanblik van een zoet slapende zoon is de grootste beloning voor mij geweest gedurende de vele extra uren werk aan dit proefschrift. Hard werken is goed en belangrijk, maar persoonlijke ontwikkeling in vrije tijd en aandacht voor mensen om jullie heen zal jullie een compleet mens maken.

Lieve Monique, het verrichten van onderzoek in de vrije tijd, wat uiteindelijk uitmondt in een proefschrift, legt een aanzienlijk beslag op de thuissituatie. Daarom wil ik jou heel erg bedanken voor de steun, het geduld en het aanhoren van de ingevingen, die vaak onder de douche of op de fiets ontstonden. Ik wens jou veel plezier op het door jou felbegeerde plekje links vooraan en hoop op vele liefdevolle, gezellige en interessante jaren tesamen met jou en onze kinderen.

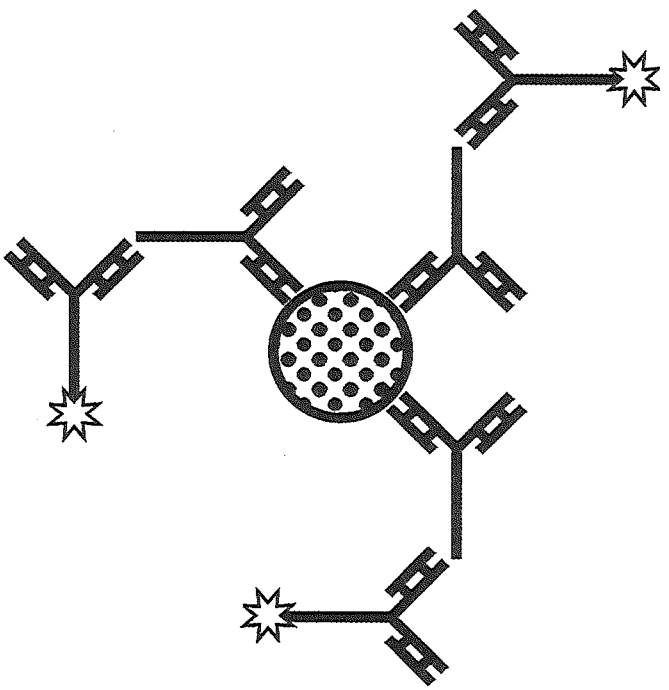
Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 14 april 1972 te Rotterdam, alwaar hij zijn middelbare schooltijd doorbracht op het Erasmiaans Gymnasium en in 1990 het gymnasium B diploma behaalde. In 1990 begon hij met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Een keuze-onderwijs op de afdeling hematologie motiveerde hem om te participeren aan een tweeledig keuze-onderzoek in 1994 in de Dr. Daniel den Hoed kliniek onder begeleiding van Dr. A. Hagenbeek en Dr. M. B. van 't Veer. Een van deze twee keuze-onderzoeken ging over de waarde van IgM antistoffen tegen trombocyten, waarmee zijn interesse in de betekenis van alloantistoffen tegen trombocyten is begonnen. Hij verrichtte zijn co-schappen in 1995 en 1996 in de regio Rotterdam, welke uiteindelijk cum laude werden afgesloten. Vanaf eind 1996 was hij werkzaam op de afdeling inwendige geneeskunde in het Zuider Ziekenhuis als arts-assistent. In juli 1997 werd hij toegelaten tot de opleiding inwendige geneeskunde (opleider dr. A. Berghout), waarvan de eerste 4 jaar werden doorgebracht op de verschillende afdelingen in het Medisch Centrum Rijnmond Zuid locatie Zuider (voorheen Zuider Ziekenhuis). De opleiding tot internist werd in juli 2001 voortgezet in het ErasmusMC (opleider prof. dr. H. A. P. Pols), alwaar hij verschillende stages verrichtte. De opleiding tot hematoloog (opleider prof. dr. B. Löwenberg), startte in het laatste jaar van de opleiding tot internist en vond plaats van september 2002 tot september 2004 in het ErasmusMC (centrum lokatie en Daniel den Hoed). Vanaf september 2004 is hij als hematoloog verbonden aan de afdeling hematologie van het ErasmusMC.

Naast de bovengenoemde werkzaamheden hebben de inspanningen als onderzoeker, die hebben geleid tot dit proefschrift, vanaf 1994 voornamelijk in de avonduren, weekenden, compensatiedagen en vakanties plaatsgevonden.

Chapter 13

List of publications



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Levin M.-D., van der Holt B., de Veld J.C., Gratama J.W., de Vries W., van 't Veer M.B. Visual scoring versus histogram subtraction of in vivo binding of immunoglobulins against platelets after transfusion. *Clinical Cytometry* 2004 57B:40-46

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Levin M.-D., van der Holt B., de Veld J.C., Gratama J.W., de Vries W., van 't Veer M.B. The value of crossmatch tests and panel tests as a screening tool to predict the outcome of platelet transfusion in a non-selected hematological patient population (*in press at Vox Sanguinis*)

Koudstaal M.J., van der Wal K.G.H., Lam K.H., Meeuwis C.A., Speleman L., **Levin M.-D.** Granulocytic sarcoma (chloroma) of the oral cavity: report of a case and literature review *(submitted)*

Levin M.-D., Graveland W.J., van 't Veer M.B., Kappers-Klunne M.C. Respons na rituximab bij patiënten met recidief idiopathische thrombocytopenische purpura: een meta-analyse van fase I/II studies (*submitted*)

den Hollander J.G., **Levin M.-D.**, van Arkel C., Lugtenburg E.J., de Marie S. Hepatotoxicity of voriconazole during treatment of suspected invasive aspergillosis in patients treated for a hematologic malignancy (*in writing*)