PLATELET TRANSFUSION AND ALLOIMMUNIZATION

CLINICAL AND LABORATORY STUDIES

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of platelet transfusion therapy

Thrombocytopenia as a result of decreased platelet production may be treated with platelet transfusions. Decreased bone marrow function resulting in thrombocytopenia may be seen in hematopoietic diseases as leukemia, myelodysplastic syndrome or conditions of infiltration of bone marrow with solid tumor metastases. Thrombocytopenia also is a frequent side effect of cancer therapy. The application of high doses of chemotherapy and/or radiotherapy may result in periods of severe bone marrow suppression. This may be more outspoken when the bone marrow function is already compromised by the underlying disease of previous cytotoxic treatment, resulting in periods of life-threatening thrombocytopenia.
Platelets play a major role in primary hemostasis. Severe thrombocytopenia increases the risk of bleeding. When the platelet count declines below $100x10^9$/L, the probability of hemorrhage increases progressively. Severe thrombocytopenia with platelet counts of less than $10x10^9$/L is associated with the occurrence of frequent bleeding[1,2].
Introduction of platelet transfusion therapy reduced hemorrhage as a major cause of death from 67% to 37% in a study of 414 patients with acute leukemia[3]. Also, an autopsy study in 57 patients with acute leukemia showed a marked decrease of bleeding as the cause of death following initiating platelet transfusion therapy, i.e. from 63% to 15%[4]. These observations have established the clinical effectiveness of platelet transfusions and have led to the administration of prophylactic platelet transfusions in patients who have to be protected during episodes of thrombocytopenia. Based on the relationship between the level of thrombocytopenia and the incidence of bleeding as described in a study in the pre-platelet transfusion days[1], platelet levels are generally maintained above $5\text{-}20x10^9$/L for prophylaxis of hemorrhage[2,5].

1.2 Refractoriness to platelet transfusions

A successful platelet transfusion results in a satisfactory post-transfusion platelet increment in the patient. Several formulas are being used to estimate corrected platelet increments or platelet recoveries following platelet

transfusions in individual patients. These formulas take into account the dose of platelets administered and the body surface area or blood volume of the patient.
Using the formula for calculation of post-transfusion platelet recovery without correction for splenic pooling i.e.

$$\text{Recovery (\%)} = 100 \times \frac{\text{absolute increment x blood volume}}{\text{no. of transfused platelets}}$$

a transfusion is considered successful when the 1hr-posttranfusion platelet recovery is more than 20% and the 16-20 hr-posttransfusion platelet recovery more than 10%. Not all transfusions are successful. When two consecutive platelet transfusions do not lead to adequate results generally the patient is considered clinically refractory.
Refractoriness may result from alloimmunization of the recipient to antigens present on donor platelets or from the presence of clinical factors that lead to decreased survival of transfused platelets such as fever, septicemia, splenomegaly, disseminated intravascular coagulation, bone marrow transplantation and administration of amphotericin B[6-10].

1.3 Alloimmunization to platelets

A variety of antibodies may be involved in patients alloimmunized against platelets. These include HLA-antibodies, platelet-specific antibodies, ABO antibodies, drug-related antibodies and antibodies directed against complement factors.
Platelets carry HLA Class I antigens and the role of HLA-antibodies in platelet refractoriness has already been established in 1965 when Bosch et al. for the first time described satisfactory platelet increments following transfusion of platelets obtained from leukocyte-group compatible relatives into a refractory patient[11]. In 1969 Yankee et al. reported in more detail on HLA matching in platelet transfusion. In 5 alloimmunized patients transfusions from HLA-matched siblings were successful, while platelets from HLA-mismatched relatives failed[12]. From further studies it has become

evident that the vast majority of cases of alloimmunization against platelets are caused by HLA antibodies.

Platelets also carry antigens that are differentiation lineage specific and not expressed on other types of hematopoietic cells[13]. The role of these so-called platelet-specific antigens and their respective antibodies is well established in the context of neonatal alloimmune thrombocytopenia and post-transfusion thrombocytopenic purpura. The frequency of platelet specific antibodies in patients receiving platelet transfusions is not precisely known but estimated to be approximately 5-15%, often in combination with HLA-antibodies[14].

In a minority of refractory patients antibodies other than HLA-antibodies and platelet-specific antibodies are involved. It has been documented that the presence of high-titered ABO-antibodies may result in rapid destruction of ABO-incompatible platelets[15]. Also, in some instances the presence of drugrelated antibodies has been shown to be the cause of platelet refractoriness[16,17]. More recently it has been suggested that antibodies against complement factors may also be involved in immunological platelet destruction[18].

All types of antibodies mentioned above may interfere with the survival of tranfused platelets and therefore the effectiveness of platelet transfusions. In most, i.e. in approximately 90%, of cases however, HLA-antibodies are involved. Yet, to permit adequate transfusion results in these alloimmunized individuals HLA-compatible platelet transfusions may be used. Although these transfusions from HLA-matched donors may still produce satisfactory results, one should be aware that several aspects make the transfusion of HLA-matched platelets cumbersome.

Firstly, one needs to find HLA-compatible donors for a patient. Due to the high degree of polymorphism of the HLA-system a large pool of HLA-typed platelet donors needs to be available to find a HLA-compatible donor for a given patient with reasonable probability. Although platelets carry only the HLA-Class I antigens, an estimated donor pool of 2500-5000 HLA-typed donors is required to identify an average number of 1-2 HLA-identical platelet donors per patient in the USA[19,20]. When cross-reactive antigens are taken into consideration the size of the pool may be somewhat smaller. It has been calculated that in the Dutch population a pool size of 2000 donors would result in the presence of 6 HLA-compatible donors for 50% of the

patients. When cross-reactive mismatches are included this number increases to 12 donors found for 80% of the patients[21]. Based on these estimations a donor pool size of 2000 HLA-typed donors was recommended for a blood bank taking care for a bone marrow transplant center. Clearly, even with donor pools of this size suitable donors are not always found for patients with rare HLA-antigen combinations.

Secondly, plateletapheresis is necessary to obtain sufficient numbers of platelets from a HLA-matched donor. This procedure poses a burden on the blood donor as it is significantly more time consuming than a routine blood donation. Further, apheresis procedures may lead to side effects due to citrate intoxication.

A third limitation to be considered is that not all HLA-matched transfusions result in successful transfusions. This may be due to the fact that a perfectly matched donor cannot be found or due to the simultaneous presence of non HLA platelet reactive antibodies such as against platelet specific antigens. McFarland et al. found that 21 out of 61 (34%) HLA-matched platelet transfusions into alloimmunized patients were not successful[8]. When transfusions in the presence of clinical factors (bleeding, sepsis, infections, splenomegaly and fever) were excluded, still 15 out of 43 transfusions (35%) were not successful. Also, in another study it was documented that 18 out of 61 (30%) well matched transfusions failed to give a satisfactory increment. In this study it was shown that the addition of a platelet crossmatch could have prevented these failures, indicating the presence of antibodies against non HLA platelet antigens[22].

To study the predictive value of the flow cytometric platelet immunofluorescence crossmatch test, as described in Chapter 7, for HLA-matched platelet transfusions into alloimmunized patients, we have analyzed both clinical and immunological factors and their correlation with the transfusion outcome in a cohort of alloimmunized patients. (Chapter 8).

1.4 Prevention of alloimmunization

Because of the problems associated with the transfusion support of HLA-immunized patients there have been many efforts to prevent sensitization rather than circumventing the problem once it has developed. In the beginning of the era of platelet transfusions the platelet products were prepared from 4-8 units of whole blood and no attempts were made to prepare the platelet concentrates without contamination of leukocytes. Typically, these products contained on average 350-400x10^9 platelets and 1-2x10^9 leukocytes per transfusion[23-25]. Also, in the majority of centers red cell concentrates were applied without additional measures for the removal of leukocytes; a red cell concentrate therefore contained approximately 1-3x10^9 leukocytes.

Strategies that were pursued to prevent alloimmunization have been directed at :

a: reduction of the number of different donors to whom the recipient was exposed to postpone the moment of alloimmunization. This was achieved by using random single donor platelet transfusions prepared by hemapheresis-procedures for platelet collection. In animal and clinical studies it became clear that the strict application of random single donor platelet products could postpone the occurrence of allo-immunization[26-28].

b: inactivation of the cells responsible for the induction if the alloimmune response by using UV-irradiated platelet transfusions[29,30].

c: several other approaches have been tested, some only experimentally in animal studies e.g. cyclosporin A, others e.g. suppression of the recipient immune system were found associated with side effects[27].

d: the most successful approach to prevent alloimmunization appears to be the reduction of the number of leukocytes contaminating the transfusion products. This method implies the use of both leukocyte-depleted platelet and red cell transfusions. Observations in mice and rats have indicated that "pure" platelets do not provoke a primary immune response[31,32]. This suggested that primary HLA-alloimmunization following platelet transfusions is caused by the

leukocytes contaminating the platelet products. On the basis of these findings patients were transfused with leukocyte-depleted platelet and red cell concentrates. The number of leukocytes in the platelet products was reduced to less than $5x10^6$. The number of leukocytes of the filtered red cell products was not specified. The incidence of alloimmunization in a group of 68 patients following leukocyte-poor transfusions was 24%. This value was much lower than the 97% frequency of alloimmunization observed among a group of 28 patients transfused with non-leukocyte depleted platelets[24]. In addition to the latter retrospective comparison, in further prospective[14] and randomized[33,34] studies, leukocyte depletion of platelets was confirmed to decreased the frequency of alloimmunization. This appeared to be true in particular with regard to the primary HLA-alloimmunization. The incidence of primary anti-HLA Class I immunization was reduced to 7 percent in multitransfused subjects[35]. However, the frequency of refractoriness in patients who had a history of previous pregnancies remained as high as 27% even when the number of leukocytes in the platelet products was reduced to $<20x10^6$ [14]. This suggests that a secondary (booster) anti-HLA immune response could not be prevented by this degree of leukocyte contamination.

Secondary HLA-alloimmunization, e.g., following prior pregnancies and subsequent boosting by transfusions, represent an important transfusion problem. It is has not been settled if a further reduction of the numbers of leukocytes contaminating the blood products would prevent the secondary alloimmunization. We initiated a randomized prospective study to investigate whether secondary HLA-alloimmunization could be prevented by leukocyte depletion of blood products to less than $5x10^6$ per transfusion (Chapter 2).

Technical aspects of leukocyte-depletion of platelet products

Early attempts to remove leukocytes from platelet suspensions used differential centrifugation techniques. These techniques have some major disadvantages. Firstly, removal of leukocytes is associated with an average

platelet loss of approximately 25%[24,25]. A second disadvantage is that centrifugation techniques are time-consuming. These are also sensitive to technician variation, which may reduce the reproducibility of the results. Thirdly, as the integrity of the closed system cannot be maintained, the storage period is reduced to 6 hours post-centrifugation at maximum.
These considerations made the differential centrifugation technique less suitable for routine application. Recently, several types of filters have been developed that allow for a fast, efficient and reproducible removal of leukocytes from the platelet products[36]. The filtration process can be performed in a closed system thus maintaining the sterility of the product.

We were interested to know the relative performances of 4 presently commercially available filtertypes i.e. Pall PL100S, Pall PL50S, Sepacell PL-10N and Sepacell PL-5N. A comparative study with regard to the in vitro characteristics of these 4 filter types is described in Chapter 3.

1.5 Detection of alloimmunization

The presence of alloantibodies in the transfusion recipient directed against antigens present on platelets is a major cause of platelet refractoriness.
However, refractoriness can also be caused by a variety of other factors (see paragraph 1.2). To establish the cause of platelet refractoriness it would be clinically important to be able to determine the presence of platelet-antibodies with techniques that have a high sensitivity and specificity and that produce a rapid result. Furthermore, it would be useful if these methods could also be used as a platelet crossmatch test to select compatible platelet donors for alloimmunized patients or to evaluate the serological compatibility of (HLA-) matched platelet donors.
A large number of tests have been developed to detect the presence of HLA- and platelet-specific alloantibodies.
The lymphocytotoxicity test (LCT) is the standard assay for HLA-typing and has been extensively applied for testing of HLA-antibodies[37]. Disadvantages are that by using lymphocytes as target cells antibodies directed at platelet-

specific antigens may not be detected and that the LCT is not very sensitive so that false negative results are frequently obtained. The sensitivity of the LCT can be increased by application of fluorescent dyes and automated reading instead of the standard method using eosine staining manual reading[38]. By using an indirect lymphocyte immunofluorescence assay the sensitivity can be increased considerably and this approach will also allow the detection of non-complement activating antibodies[39]. However, by nature even these tests will not allow the detection of antibodies against platelet specific antigens and may therefore be falsely negative as assays for platelet sensitization. Therefore, tests using platelets as target cells are needed in addition. In the early days, tests for the detection of platelet antibodies were based on "imitation" of red cell antibody tests such as the platelet agglutination assay, or were based on measurement of alteration/activation of platelet function upon incubation of test platelets with patient serum e.g. platelet factor 3 assay, serotonin release and uptake, adenine release, ^{51}Cr release, platelet aggregometry and inhibition of platelet migration. These tests were cumbersome and difficult to standardize with variable (poor) reproducibility[40].

Current methods focus on the detection of platelet-bound immunoglobulins. In general, the amount of immunoglobulins bound to the test-platelets following incubation with the test-serum is determined by incubation with a second anti-immunoglobulin antibody that has been labeled by enzymes, immunofluorescent molecules or radioactivity - to allow quantification.

During recent years we have evaluated the applicability of several different assays for the detection of antibodies directed against platelets.

- A ^{51}Cr-platelet lysis assay (Chapter 4)
- An ELISA using solid-phase platelet monolayers (Chapter 5)
- An immunofluorescence technique allowing simultaneous detection of antibodies against platelets, granulocytes and lymphocytes using flow cytometric analysis (Chapter 6)
- A commercially available platelet antibody assay which was compared with the ELISA and immunofluorescence assay (Chapter 7).

1.6 Aim of the study

The first part of this thesis deals with the prevention of secondary HLA-alloimmunization and the technical approaches of leukocyte depletion of platelet transfusions.
The following questions were studied:

1. Is it possible to prevent secondary HLA-alloimmunization by leukocyte depletion of platelet transfusions to less than $5x10^6$ leukocytes per transfusion?
   (Chapter 2)

2. How does the qualitative performance of different recently developed filter types compare with regard to the extent of leukocyte depletion of platelet products?
   (Chapter 3)

Clinical platelet refractoriness may result from antibodies directed against platelets. Especially when such common clinical conditions (e.g. septicemia, splenomegaly, disseminated intravascular coagulation) inhibit the efficiency of platelet transfusions, serological tests to identify serum antibodies are needed to establish the simultaneous presence of alloimmunization. The second part of thesis is concerned with a clinical evaluation of existing assays and the development of new assays for antibody detection.

3. Evaluation of the ^{51}Cr platelet lysis assay.
   (Chapter 4)

4. Development and evaluation of a platelet-ELISA.
   (Chapter 5)

5. Development and evaluation of an immunofluorescence assay using flow cytometric analysis to determine the presence of antibodies against platelets, granulocytes and lymphocytes simultaneously.
   (Chapter 6)

6. Evaluation of a commercially available assay, the Modified Capture-P assay, in comparison to the platelet ELISA and the flow cytometric immunofluorescence test.
(Chapter 7)

Platelets from HLA-matched donors are usually searched and prepared as transfusion products for alloimmunized patients. However, these HLA-matched transfusions are not always successful e.g., when refractoriness to platelet transfusions is caused by the simultaneous presence of platelet-specific antibodies. In the final part of this thesis we wished to evaluate the performance of the flow cytometric platelet immunofluorescence test as a platelet crossmatch for the prediction of the platelet transfusion result

7. To study the predictive value of a platelet immunofluorescence crossmatch for HLA-matched platelet transfusions into alloimmunized patients, considering the effects of both clinical and immunological factors on the transfusion outcome in a cohort of alloimmunized patients.
(Chapter 8).

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CHAPTER 2

LEUKOCYTE DEPLETION OF RANDOM SINGLE - DONOR PLATELET TRANSFUSIONS DOES NOT PREVENT SECONDARY HUMAN LEUKOCYTE ANTIGEN - ALLOIMMUNIZATION AND REFRACTORINESS: A RANDOMIZED PROSPECTIVE STUDY

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Leukocyte Depletion of Random Single-Donor Platelet Transfusions Does Not Prevent Secondary Human Leukocyte Antigen–Alloimmunization and Refractoriness: A Randomized Prospective Study

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We studied the value of leukocyte depletion of platelet transfusions for the prevention of secondary human leukocyte antigen (HLA)-alloimmunization in patients with a high-risk of prior immunization induced by pregnancies. Seventy-five female patients with hematologic malignancies (mostly acute leukemia) and a history of pregnancy were randomized to receive either standard random single-donor platelet transfusions (mean leukocytes, 430 × 10^6 per transfusion) or leukocyte-depleted random single-donor platelet transfusions. Leukocyte depletion to less than 5 × 10^6 leukocytes per platelet transfusion (mean leukocytes, 2 × 10^6 per transfusion) was achieved by filtration. Of the 62 evaluable patients, refractoriness to random donor platelets occurred in 41% (14 of 34) of the patients in the standard group and in 29% (8 of 28) of the patients in the filtered group (P = .52); anti-HLA antibodies developed in 43% (9 of 21) of individuals in the standard group and 44% (11 of 25) of cases in the filtered group. The time toward refractoriness and development of anti-HLA antibodies was similar for both groups. We conclude that leukocyte depletion of random single-donor platelet products to less than 5 × 10^6 per transfusion does not reduce the incidence of refractoriness to random donor platelet transfusions because of boostering of anti-HLA antibodies.

REFRACTORINESS to random donor platelets caused by alloimmunization occurs in 30% to 50% of patients.[1-3] The antibodies involved are mostly directed against human leukocyte antigens (HLAs). Procedures to provide alloimmunized patients with platelets obtained from HLA-matched donors[4] or alternatively from donors selected by a platelet crossmatch[5] are logistically complicated, costly, and still 10% to 30% of these transfusions are unsuccessful.[6] This explains the interest for developing methods to prevent alloimmunization.

Studies both in rats and in mice have shown that pure platelets do not provoke a primary immune response.[7,8] This suggests that primary HLA-alloimmunization after platelet transfusions is caused by the leukocytes contaminating the platelet suspension. A nonrandomized retrospective study in humans indeed suggested that leukocyte depletion of platelet transfusions reduced the development of HLA-alloimmunization.[9] In further randomized and/or prospective studies, it was confirmed that leukocyte depletion of both platelet transfusions and red blood cell (RBC) products resulted in a decreased frequency of HLA-alloimmunization,[2,3,10,11] in particular, the incidence of the primary anti-HLA immune response.[11,12] In a nonrandomized prospective study on the value of leukocyte-depleted multiple donor platelet transfusions containing less than 20 × 10^6 leukocytes per transfusion, it was observed that the incidence of HLA-alloimmunization was not decreased in patients with previous pregnancies in contrast with nonpresensitized patients.[10] This suggests that a secondary (or booster) anti-HLA immune response occurred that was not prevented by the degree of leukocyte depletion applied.

It is the aim of the present study to investigate the role of more vigorous leukocyte depletion of platelet suspensions, ie, less than 5 × 10^6 leukocytes per transfusion for the prevention of the secondary anti-HLA immune response. Therefore, only females with previous pregnancies were the subjects of a prospective randomized controlled study that was conducted in four centers. Random single-donor platelet transfusions were used exclusively because it has been documented that the use of this non–leukocyte-depleted product might postpone the development of alloimmunization,[13,14] especially in patients with previous pregnancies.[14]

MATERIALS AND METHODS

Patients

Patients were eligible for entry into the study when they fulfilled all of the following criteria: (1) untreated hematologic malignancy; (2) history of previous pregnancies; (3) no blood transfusions received in the past 6 weeks except for filtered RBCs; and (4) transient thrombocytopenia caused by bone marrow (BM) failure. Seventy-five patients were randomized and stratified by hospital to receive either standard random single-donor platelets (standard group) or random single-donor platelets leukocyte depleted by filtration (filtered group). Patients remained on study until one of the following events occurred: (1) refractoriness to random single-donor platelets; (2) death; or (3) BM transplantation.

Preparation of Blood Components and Transfusion Policy

RBC concentrates. Buffy-coat–depleted RBC concentrates were filtered within 36 hours after collection through a cellulose acetate filter (Cellselect; Nederlands Produktielaboratorium voor Bloedtransfusieapparatuur en Infusievloeistoffen BV, Emmer-Compascuum, The Netherlands) in a closed system using a sterile docking device (SCD; Haemonetics, Braintree, MA). This procedure results in a median number of 0.4 × 10^6 leukocytes/U with 99% of units containing less than 5 × 10^6 leukocytes.[15]

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Table 1. Patient Characteristics

	Standard Group	Filtered Group
Total no. of evaluable patients	34	28
Mean age (range)	50 (18-74)	50 (33-76)
Diagnosis		
ANLL	27	22
ALL	6	2
NHL	1	1
MDS	0	2
MM	0	1
Mean no. of pregnancies (range)	3 (1-9)	3 (1-11)
No. of previously transfused patients	3	5

Abbreviations: ANLL, acute nonlymphocytic leukemia; ALL, acute lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; MDS, myelodysplastic syndrome; MM, multiple myeloma.

Single-donor platelet concentrates. Single-donor platelets were collected by hemapheresis using discontinuous flow centrifugation (Haemonetics, V50, surge-protocol). Patients in the standard group received the platelet suspensions without further processing. For the preparation of leukocyte-depleted platelet products, platelet suspensions were filtered through a cellulose acetate filter (Cellselect: NPBI, Emmer-Compascuum, The Netherlands) designed for filtration of RBC. This filter has been shown to be efficient for the removal of leukocytes from platelet suspensions provided that the platelets are inactivated during filtration either by adding ACD-A to lower the pH to 6.5 to 6.8, or 12.5 μg prostacylin to the platelet suspension before filtration.[16] Filtration was performed within 36 hours after collection to reduce the risk of immunization by leukocyte fragments. After filtration, the platelets were transfused immediately. Standard platelets were also transfused within 36 hours after collection. Platelet products were routinely irradiated (20 Gy) in one center.

The platelet and leukocyte contents of standard and filtered platelet products are shown in Table 1. Platelet and leukocyte counts prefiltration were established automatically (different types of analyzers per center). Leukocyte counts postfiltration were counted manually using a 1/10 dilution of the blood sample with Türk Solution and a Fuchs Rosenthal chamber. The detection limit of this method is $\approx 5 \times 10^6$/L leukocytes, which corresponds to $\approx 2 \times 10^5$ leukocytes per platelet transfusion.

Transfusion regimen. ABO-compatible platelets were transfused prophylactically when the platelet count dropped below 10 to 20 $\times$ 10^9/L, or therapeutically for invasive procedures and in case of major hemorrhagic symptoms at platelet counts above 10 to 20 $\times$ 10^9/L. No granulocyte transfusions were administered.

Assessment of platelet recovery and definition of refractoriness. Posttransfusion platelet recoveries were performed 1 hour and 16 to 20 hours after the platelet transfusion. Platelet recovery was calculated according to the following formula:

$$\text{Recovery (\%)} = 100 \times \frac{\text{Absolute Increment} \times \text{Blood Volume}}{\text{No. of Transfused Platelets}}.$$

Blood volume was calculated as body surface area (m^2) $\times$ 2.5. No correction for splenic pooling was made.

Patients were considered refractory to random single-donor platelet transfusions when one of the following occurred: (1) two consecutive platelet transfusions with 1-hour posttransfusion platelet recoveries of less than 20% in the absence of clinical factors known to adversely affect platelet recovery (temperature > 38.5°C, septicemia, disseminated intravascular coagulation [DIC] or splenomegaly); or (2) a single platelet transfusion with 1-hour posttransfusion platelet recovery of less than 20% in the presence of anti-HLA antibodies.

Anti-HLA antibody screening. Sera were collected at entry and at weekly intervals during a transfusion period and on clinical indication, ie, in case of poor posttransfusion platelet recoveries. Final serum samples were obtained 2 to 4 weeks after the last transfusion. Sera were stored at −70°C until testing. Each serum was tested in the complement-dependent lymphocytotoxicity test[17] against a panel of 21 selected donors covering most of the defined HLA-A and HLA-B specificities. The screening test was considered positive when at least 10% of the panel cells showed cytotoxicity (>50% dead cells per well).

Statistical analysis. The occurrence of clinical refractoriness and the development of anti-HLA antibodies were analyzed as a function of time and the number of transfusions received using the actuarial method of Kaplan-Meijer. The log-rank test was applied to test for differences between the two treatment groups. Cox proportional hazard model was used to calculate the relative risk of developing refractoriness or HLA antibodies in the filtered group as compared with the standard group with 95% confidence limits (CLs). The Mann-Whitney U test was used to test for differences between the percentages panel reactive antibodies of the two groups.

RESULTS

Patients

Thirteen patients were excluded from evaluation because: (1) anti-HLA antibodies were present at entry (n = 4); (2) less than two transfusions were administered (n = 7); (3) administration of blood products other than filtered RBCs in the period of 6 weeks preceeding entry (n = 3). The characteristics of the 62 evaluable patients are shown in Table 2. No statistically significant differences were apparent between the groups with regard to age, diagnosis, number of previous pregnancies, and history of previous transfusions.

Protocol Violations

During the study period, pooled leukocyte-depleted (filtered) random multiple-donor platelet concentrates were by error administered on 14 occasions: 8 transfusions into 6 patients in the standard group; 6 transfusions into 4 patients in the filtered group. Of these 10 patients, 3 became refrac-

Table 2. Platelet Products

	Standard Group	Filtered Group
No. of products	347	324
No. of platelets ($\times 10^5$)	352 ± 94	302 ± 102
No. of leukocytes ($\times 10^6$)	430 ± 703	2 ± 3
No. of product with leukocytes:		
$<2 \times 10^6$	0 (0%)**	159 (52%)
$2\text{-}5 \times 10^6$	3 (1%)	133 (44%)
$6\text{-}10 \times 10^6$	1 (.3%)	7 (2%)
$11\text{-}20 \times 10^6$	7 (2%)	3 (1%)
$>20 \times 10^6$	317 (97%)	1 (.3%)
ND	19	21

No. of platelets and leukocytes are expressed as mean ± SD. Values in parentheses represent the percentage of counted products.

Abbreviation: ND, not determined.

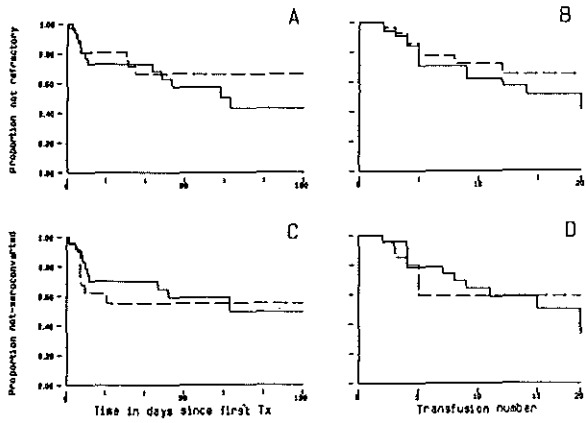

Fig 1. Kaplan-Meier life table plot of frequency and rate of refractoriness as a function of time (A) or number of platelet transfusions (B) and anti-HLA antibody formation as a function of time (C) or number of platelet transfusions (D). Ordinate: proportion of patients not becoming refractory (A, B) and proportion of patients not forming anti-HLA antibodies (C, D). (---), Filtered group; (—), standard group.

tory after this protocol violation: 2 patients in the standard group and 1 patient in the filtered group. Exclusion of these patients from analysis from the moment of receiving a random multiple-donor transfusion would not have altered the outcome of the analysis (data not shown).

Eight patients in the filtered group received a total number of 11 platelet products containing greater than 5×10^9 leukocytes. Two of these patients became refractory. The number of leukocytes in the four transfusions administered to these two patients varied from 5.1×10^9 to 7.0×10^9 leukocytes with the exception of one transfusion containing 20×10^9 leukocytes.

Development of Refractoriness and HLA-Alloimmunization

Refractoriness. In this study, 671 random single-donor transfusions were administered during the study period in both groups. Recovery data were available in 592/671 (88%) transfusions at 1 hour and in 576/671 (86%) transfusions at 16 to 20 hours posttransfusion. In 643/671 transfusions (96%), the 1- or the 16-hour recovery measurement was available.

Twenty-two of 62 (35%) patients became refractory to random single-donor platelet transfusions. The judgement of refractoriness was established as follows: (1) in 17 patients, 1-hour posttransfusion platelet recoveries of less than 20% occurred on at least two successive occasions; in 3 of these patients, nonimmunologic factors known to decrease platelet recovery (temperature >38.5°C: n = 2, DIC: n = 1) were present; in the sera of these 3 patients, anti-HLA antibodies were demonstrable. (2) in five patients, a 1-hour posttransfusion platelet recovery of less than 20% occurred once in the presence of anti-HLA antibodies.

In Fig 1, A and B, the development of refractoriness for the two groups is shown. In the standard group, 14/34 (41%) patients became refractory, versus 8/28 (29%) patients in the filtered group. Actuarial analysis using the log rank test showed that this difference was not statistically significant ($P = .52$). This type of analysis includes both the incidence of refractoriness and the time interval to develop refractoriness. When the patients in the filtered group were censored from the moment of receiving a platelet product containing more than 5×10^9 leukocytes, the difference between the two groups was also not statistically significant (log-rank $P = .32$). With the Cox proportional hazard model, using the number of days until refractoriness or until the last transfusion as time variable, the relative risk of becoming refractory in the filtered group as compared with the standard group was 0.75 (95% CL: 0.31, 1.8). This corresponds to a 9% reduction in the actuarial probability of becoming refractory (95% CL: −21%, +31%).

HLA-alloimmunization. From 55 of the 62 evaluable patients, sera were available and tested for the presence of anti-HLA antibodies. In 9 of these 55 patients, anti-HLA antibodies were present in the serum obtained at entry and these patients were not evaluable for the occurrence of seroconversion. The results of the analysis regarding the occurrence of seroconversion in the remaning 46 patients are shown in Fig 1, C and D. Anti-HLA antibodies developed in 9/21 (43%) of patients receiving filtered platelets and in 11/25 (44%) of patients receiving standard platelets. There were no statistically significant differences in both incidence of anti-HLA antibodies and time to develop anti-HLA antibodies (log-rank $P = .41$). The relative risk of developing anti-HLA antibodies in the filtered group as compared with the standard group was 1.5 (95% CL: 0.6, 3.7). This corresponds to an increase in the actuarial probability of developing anti-HLA antibodies of 14% (95% CL: −16%, +42%, Cox proportional hazard model). With regard to the reactivity pattern of the anti-HLA antibodies, the median percentage panel reactive antibodies (PRA) at the time of seroconversion in the filtered group was 24% (range, 10 to 67) and in the standard group 43% (range, 10 to 100). This difference was statistically not significant ($P = .65$, Mann-Whitney U test). Sera contained multispecific anti-HLA antibodies with the exception of sera from five patients in which HLA-specificities were identified. Sera of four patients in the filtered

group showed HLA specificities: anti-B5 (10% PRA); anti-B7 (24% PRA); anti-A2 + B44 (67% PRA); and anti-A3 + B5 (19% PRA). In the standard group, only in one patient anti-A1 was identified (52% PRA).

Of the 22 refractory patients, five were considered refractory because a single transfusion failure was associated with the presence of anti-HLA antibodies. Fifteen of the remaining 17 refractory patients were evaluable for anti-HLA antibodies: in 13 of these 15 (87%), anti-HLA antibodies were detected. In 35 of the 40 nonrefractory patients, the presence of anti-HLA antibodies could be evaluated: in 11 of these 35 patients (31%), anti-HLA antibodies were detected.

DISCUSSION

The present study shows that in patients with previous pregnancies, leukocyte depletion of random single-donor platelet transfusions to less than 5×10^6 leukocytes per transfusion does not reduce the incidence of platelet refractoriness and anti-HLA antibodies. In ≈40% of the patients studied, anti-HLA alloantibodies developed irrespective of whether platelet products were leukocyte depleted or not. There are several arguments indicating that in the majority of patients the development of anti-HLA antibodies resulted from a secondary anti-HLA immune response. Firstly, the frequency of 40% HLA-alloimmunization after transfusion of filtered blood products observed in this study is much higher than has been found in patients at risk for primary HLA-alloimmunization of whom only 11% developed anti-HLA antibodies after leukocyte-depleted transfusions.[11] Secondly, it has been shown that 40% of females in second or further pregnancy develop anti-HLA antibodies,[18,19] which is in close agreement with the frequency of anti-HLA antibodies found in this study. Thirdly, in both study groups, anti-HLA antibodies occurred in 50% of the alloimmunized patients within 2 weeks of the first transfusion. This is considerably faster than in primary HLA-alloimmunization where this took a median of 8 to 16 weeks for patients receiving filtered platelet products ($<5 \times 10^6$ leukocytes) and a median of 4 weeks for patients in the control group (mean number of leukocytes, 35×10^6).[11]

Previous studies have identified women with pregancies as patients with an increased risk for HLA-alloimmunization. When leukocyte-depleted platelet transfusions, which contain, on average, 46×10^6 leukocytes, were administered, 6 of 8 patients with previous pregnancies became refractory versus none of 17 nonpresensitized patients.[20] Previously, it had been shown that platelet transfusions containing less than 20×10^6 leukocytes induced refractoriness in 15 of 71 (21%) of females with previous pregnancies, whereas this occurred in only 16 of 264 (6%) nonpresensitized patients.[10] In the present study, leukocyte depletion was more vigourous and we have now shown that platelet products containing less than 5×10^6 leukocytes (mean, 2×10^6) do not result in a reduction of the frequency of refractoriness and HLA-alloimmunization nor in the time required to become refractory or develop HLA-alloimmunization.

An explanation for this finding may be that platelets per se are able to induce a secondary HLA-immune response. Studies in rats and mice[7,8] have suggested that this might be the case, although it cannot be excluded that small numbers of leukocytes that were below detection level have been present in the transfusion products used in these studies. To test this hypothesis, further studies are needed with blood products containing even less contaminating leukocytes, eg, less than 10^4 per transfusion. Such a study would require both the availability of new blood filters with increased leukocyte removal capacity that are currently being developed[21] and also the development of methods that allow enumeration of the very low number of leukocytes remaining postfiltration, eg, by PCR.

Also based on animal studies, soluble HLA-antigens or microparticles escaping leukocyte filtration may evoke platelet refractoriness.[22,23] In humans, Pellegrino et al[24] found that transfusion of plasma that contains leukocyte fragments from selected donors resulted in the onset of anti-HLA antibodies. As our patients received blood products that contained plasma, it cannot be excluded that this might have had a role in the stimulation of anti-HLA antibodies.

Another approach to reduce secondary HLA-alloimmunization is the use of UV-irradiation of blood products. Preliminary data from animal and clinical studies suggest that UV irradiation might reduce primary HLA-alloimmunization, while in vitro data suggest that the secondary immune response may be prevented.[25,26] It was suggested in animal studies that UV-B irradation is superior to leukocyte depletion in the prevention of the immune response.[27]

Finally, it might be worthwile to explore strategies based on other mechanisms to modify the immune response. Studies in renal transplantation patients have shown that the transfusion of blood containing leukocytes sharing at least one HLA-DR antigen between donor and recipient results in a strongly reduced incidence of primary antibody formation followed by a state of immune unresponsiveness to further mismatched transfusions.[28] Whether this phenomenon could be applied for the prevention of a secondary immune response remains to be elucidated.

In conclusion, it is now well established that primary HLA-alloimmunization caused by transfusions of RBCs and platelets can be prevented when the number of leukocytes is reduced below the threshold for alloimmunization by leukocyte depletion of the blood products. Although the number of leukocytes that will lead to a primary immune response is not precisely known, presently available blood bank technology, especially filtration, will allow the routine preparation of blood products with a degree of leukocyte depletion sufficient to prevent primary HLA-alloimmunization. A subgroup of patients now remains for whom the use of leukocyte-depleted transfusions does not reduce the incidence of alloimmunization, ie, women with previous pregnancies. Hence, future studies dealing with the prevention of transfusion-induced HLA-alloimmunization should focus on this category of patients.

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CHAPTER 3

COMPARISON OF FOUR FILTERS FOR LEUKOCYTE DEPLETION OF SINGLE DONOR PLATELETAPHERESIS PRODUCTS

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Comparison of Four Filters for Leukocyte Depletion of Single Donor Plateletapheresis Products*

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■ Removal of leukocytes from platelet suspensions is important in preventing the development of alloimmunization to HLA antigens in repeatedly transfused patients. Several types of filters have recently become available to remove leukocytes from platelet suspensions. We have compared the results obtained with Pall PL100S, Pall PL50S, Sepacell PL–10N and Sepacell PL–5N filters. Platelets were obtained by plateletapheresis using the Haemonetics V-50 TSPP surge procedure. A total of 160 platelet suspensions were studied, 40 for each type of filter used. The median number of leukocytes pre-filtration was 321×10^6 (range: 16–4068). The median number of leukocytes post-filtration was 2×10^6 (<1–521) and was dependent on the number of leukocytes present pre-filtration but not on the type of filter used. All filters tested showed approximately a 2 $\log_{10}$ reduction of leukocytes. When the number of leukocytes was below 500×10^6 pre-filtration, the number of leukocytes post-filtration did not exceed 10×10^6. Overall platelet loss was 11±7% and was lowest with the Pall PL50S filters (7±7%). Mean platelet volumes post-filtration were decreased with all types of filters used. ■

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INTRODUCTION

A major problem in platelet transfusion therapy is the development of alloimmunization to HLA antigens in 40–70% of repeatedly transfused patients.[1,2] This appears to be caused by the presence of leukocytes in the platelet suspensions as the strict use of leukocyte depleted platelet suspensions decreases the frequency of HLA alloimmunization to 10–15%[3–5]. For application in routine use a simple method is needed for efficient and reliable leukocyte depletion of platelet suspensions. Several types of filters have recently been developed for this purpose. In this study we compared the *in vitro* performances of 4 different filter systems for the removal of leukocytes from single donor plateletapheresis products.

MATERIALS AND METHODS

Platelets were collected from healthy donors using the Haemonetics V50 cell separator and the time saver protocol (TSPP, Haemonetics, Braintree, Mass. U.S.A.). The following platelet filters were used: Pall PL100S and Pall PL50S (Pall Corporation, Glen Cove, N.Y., U.S.A.); Sepacell PL–10N and Sepacell PL–5N (Asahi, Tokyo, Japan). Forty filtrations were carried out with each type of filter. The filtrations were performed 24±2 (mean±SD) min following the end of the final collection cycle of the plateletapheresis procedure. The flow rate of the platelet suspension through

the filter was controlled at 19±4 mL/min (range 11–33) using a roller clamp. To monitor the flow rate, a drip chamber was assembled in the tubing in front of the filter. Filters were not primed as stated in the manufacturer's instructions. All connections were made using a sterile docking device (SCD 312, Haemonetics, Braintree, Mass. U.S.A.). Platelet counts and mean platelet volumes (MPV) were determined using an electronic cell counter (H1, Technicon, Gorinchem, Holland). Pre-filtration, the leukocytes were also counted electronically. The leukocytes were counted manually post-filtration. For this, the blood sample was diluted with 9 vols of Türk's solution. A Fuchs–Rosenthal counting chamber (total volume counted 6.4 µL) was used. The lower detection limit of this counting procedure is 5×10^6 leukocytes per liter, as the volume of the platelet products varied from 169 to 456 mL, this resulted in a lower detection limit of approximately 1.5×10^6 leukocytes per platelet suspension. pH was determined using a blood gas analyzer (BGM 1312, Instrumentation Laboratory Inc., Lexington, Mass. U.S.A.).

STATISTICAL ANALYSIS

For each filter the leukocyte depletion was analyzed by graphically comparing the total number of leukocytes post- and pre-filtration and by calculating the average log reduction. The $\log_{10}$ reduction was defined as $\log_{10}$ (number of leukocytes pre-filtration / number of leukocytes post-filtration). If no leukocytes were counted post-filtration (n = 13), the number of leukocytes post-filtration was estimated by 0.001×volume of suspension post-filtration. Analysis of variance and covariance was applied to test for differences between filters and for association between the number of leukocytes pre- and post-filtration. The same techniques were used to study platelet loss and changes in MPV and pH. Means were reported ± standard deviation.

RESULTS

Platelet suspensions obtained by plateletapheresis (n=160) contained a median (range) of 390 (232–647)$\times10^9$ platelets and 321 (16–4068)$\times10^6$ leukocytes in a collection volume of 300 (169–453) mL.

The results of leukocyte depletion for each filter are shown in Fig. 1. The median number of leukocytes in the platelet suspension post-filtration was 1.6×10^6 (<1–521) and was strongly dependent on the number of leukocytes present pre-filtration. Relatively high numbers of leukocytes post-filtration (>10×10^6) (n=27) were found only when the number of leukocytes pre-filtration was >500×10^6 (n=58). Mean $\log_{10}$ reduction for all filters was 2.1±0.6 and was not different for the different types of filters used (P>0.20).

Platelet loss following filtration was lowest with the Pall PL50S filters (mean loss: 7.4%) and significantly lower (ANOVA P<0.001) than with the Pall PL100S, Sepacell PL–10N and Sepacell PL–5N filters (mean loss 11.0–13.4%) (Table 1). The mean volume loss of the Pall PL50S was 13.6 mL and of the other filters was 19.3–19.5 mL (P>0.001) (Table 2). Platelet loss was greater than that calculated on the basis of volume loss in the dead space of the filter alone, indicating trapping of platelets in the filter. The numbers of platelets trapped were lower with both types of Pall filters than with the Sepacell filters (P>0.003). A positive correlation was observed between the numbers of platelets trapped and the concentration of leukocytes (P>0.001) and platelets (P=0.03) in the platelet suspension pre-filtration.

MPV pre-filtration was 8.8±0.7 fL. Following filtration a statistically significant decrease of 0.28±0.33 fL (data for all filters) was observed (Table 3). The decrease in MPV was greater with the Sepacell filters than with the Pall filters, but the difference between the filters was not statistically significant (P=0.08).

pH pre-filtration was 7.00±0.05. Following filtration there was a slight but statistically significant increase in pH

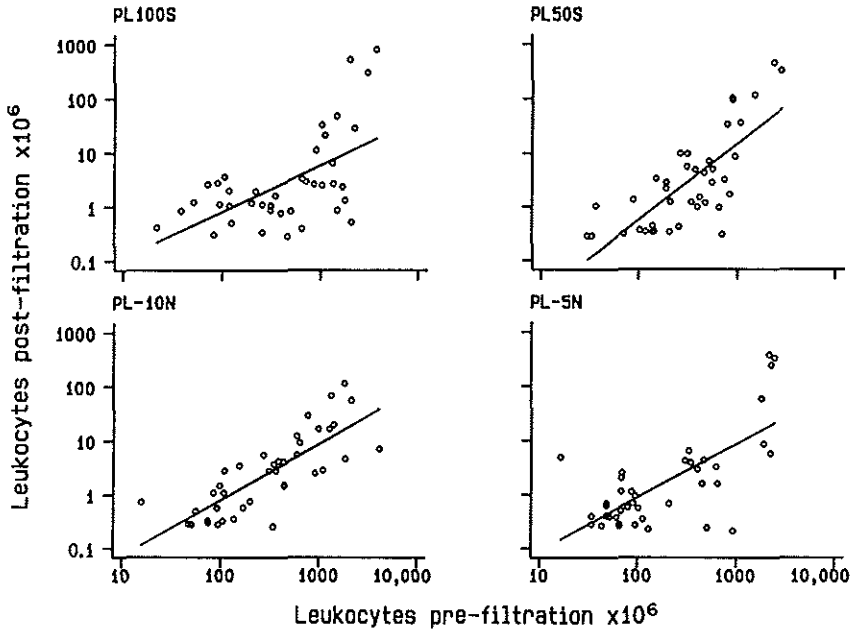

Figure 1. Comparison of leukocyte-depletion capacities of four types of filters: Pall PL100S; Pall PL50S; Sepacell PL–10N and Sepacell PL–5N. Using each type of filter 40 filtrations were performed. Solid lines indicate regression lines, fitted separately for each filter. Regression lines were not statistically significantly different between the filters.

Table 1. Platelet Loss Due to Filtration for Leukocyte Depletion of Platelet Suspensions

Filter	No of Platelets ($\times 10^9$) Pre*	Post†	Platelet Loss (%)	Platelets Trapped‡ (%)
PL100S	411±78§	368±81	11.0±7.1	4.5±6.2
PL50S	403±61	372±57	7.4±6.7	2.9±5.0
PL–10N	402±81	353±80	12.2±5.7	6.1±5.2
PL–5N	375±75	320±64	13.4±4.8	6.7±4.2

PL100S = Pall PL100S; PL50S = Pall PL50S; PL–10N = Sepacell PL–10N; PL–5N = Sepacell PL–5N.
*Pre-filtration.
†Post-filtration.
‡Platelets trapped = the platelet loss exceeding that caused by loss of the platelet suspension in the dead space of the filter.
§Mean±SD.

with the Pall PL50S, Sepacell PL–10N and the Sepacell PL–5N filters (Table 3).

DISCUSSION

Platelet suspensions obtained by plateletapheresis using the Haemonetics V50 cell separator and the time saver protocol showed a wide variation in leukocyte contamination (range 16–4068×10^6 leukocytes). Therefore, these products were informative in establishing the performance of filters for leukocyte depletion of platelet suspensions.

Table 2. Volume (Dead Space) of Filters for Leukocyte Depletion of Platelet Suspensions

Filter	Volume of Suspensions Pre-filtration (mL)	Volume Loss (mL)	Volume Loss (%)
PL100S	309±55*	19.5± 4.5	6.5±1.9
PL50S	318±68	13.6±10.2	4.7±4.2
PL–10N	322±58	19.4± 2.5	6.2±1.4
PL–5N	297±51	19.3± 1.9	6.6±1.3

PL100S = Pall PL100S; PL50S = Pall PL50S; PL–10N = Sepacell PL–10N; PL–5N = Sepacell PL–5N.
*Mean ± SD.

Table 3. Changes in Mean Platelet Volume and pH Following Filtration

Filter	MPV (fL)*	pH*
PL100S	−0.20±0.33†	0.005±0.029
PL50S	−0.23±0.30	0.012±0.020
PL–10N	−0.31±0.37	0.030±0.032
PL–5N	−0.38±0.31	0.042±0.039
Total	−0.28±0.35‡	0.022±0.034‡

PL100S = Pall PL100S; PL50S = Pall PL50S; PL–10N = Sepacell PL–10N; PL–5N = Sepacell PL–5N.
*Post-filtration – pre-filtration values.
†Mean ± SD.
‡$P<0.001$, paired t-test.

A problem in studying leukocyte depletion is the counting of the low number of leukocytes post-filtration which electronic counting devices cannot reliably achieve. Therefore, we used a manual counting method using a large counting chamber (6.4 µL) and a 1:10 dilution of the sample before counting, resulting in a lower detection limit of 5×10^6 leukocytes per liter.

To prevent HLA alloimmunization, the number of leukocytes present in the platelet suspension must be reduced to below the level needed to induce formation of antibodies. The precise dose of leukocytes required for the development of HLA-alloimmunization is unknown but may be estimated to be in the order of $15–50\times10^6$ leukocytes.[5,6] For all filters tested on average a 2.1 $\log_{10}$ reduction was observed. This is in agreement with existing data on Pall PL100,[7–9] Sepacell PL–10N[10] and Sepacell PL–5A[11] filters. For Sepacell PL–10A a 3.5 $\log_{10}$ reduction was reported.[7] We showed that all filters tested produced platelet products with leukocyte numbers $<10\times10^6$ post-filtration when the number of leukocytes pre-filtration did not exceed 500×10^6. Similar observations have been reported with the Pall 100S filters.[9] We did not find any statistically significant differences in the leukocyte depletion capacities between the various types of filters.

The platelet suspensions were filtered almost immediately (22 min) following collection. This resulted in an average platelet loss of 11% for all filtrations performed. Thus, there is no need to wait any longer after the collection and this finding suggests that on-line filtration would be feasible. Platelet loss was lowest with the Pall PL50S filter because this filter has a smaller volume than the other filters tested. Further, it appeared that less platelets were trapped in the Pall filters than in the Sepacell filters tested, although this effect was rather small.

Mean platelet volumes post-filtration were statistically significantly lower than pre-filtration for all filters tested, indicating selective removal of the larger platelets. Similar observations were reported for the Pall PL100 and Sepacell PL–10A filters.[7,12]

For three types of filters (Pall PL50S, Sepacell PL–10N and Sepacell PL–5N) a small, but unexplained statistically significant increase in mean pH post-filtration of 0.02–0.04 was found.

In conclusion, all filters tested were easy to use and showed efficient leukocyte removal resulting in less than 10×10^6 leukocytes post-filtration when the number of leukocytes pre-filtration was below 500×10^6. There was minimal platelet loss using the Pall PL50S filter.

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CHAPTER 4

THE VALUE OF A ^{51}CR PLATELET LYSIS ASSAY AS CROSSMATCH TEST IN PATIENTS WITH LEUKAEMIA ON PLATELET TRANSFUSION THERAPY

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The value of a ^{51}Cr platelet lysis assay as crossmatch test in patients with leukaemia on platelet transfusion therapy

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SUMMARY. Current platelet crossmatch testing still results in a significant percentage of false positive or false negative results in oncological patients during platelet support. A ^{51}Cr platelet lysis assay was used for the detection of platelet alloantibodies. We determined the predictive value of this assay as crossmatch procedure on 28 occasions in 14 patients who received random single donor platelet transfusions. To deal with the problem of spontaneous lysis we included a panel of 10 control sera from normal individuals and applied several methods of statistical analysis to these data. It appeared that the use of only one control serum was sufficient when the percentage relative counts was used as the criterion variable, which is of advantage in the practical application of the test. The test values were retrospectively compared with the clinical transfusion response, determined as the 1 h post-transfusion platelet recovery. The ^{51}Cr platelet lysis crossmatch showed false negative results in 1/21 cases and false positive results in 1/7 cases. These data indicate that the ^{51}Cr platelet lysis assay adds a useful dimension to the solution of the problem of selecting compatible platelet donors. Spontaneous lysis of target platelets appeared not to be a problem in the interpretation of test results.

Refractoriness due to alloimmunization is a major problem in the platelet supportive care of cancer patients. A variety of platelet crossmatch tests has been developed for the *in vitro* prediction of the platelet transfusion outcome. However, in a considerable number of patients the transfusion result cannot be predicted with a single test because of false positive and false negative results (Filip *et al*, 1976; Gmür *et al*, 1978; Brand *et al*, 1978; Tosato *et al*, 1980; Meyers *et al*, 1981; Waters *et al*, 1981; Kickler *et al*, 1983). This indicates the need for more reliable crossmatch tests. Cimo & Gerber (1979) used a ^{51}Cr platelet lysis assay, as originally

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developed by Aster & Enright (1969) for the detection of platelet alloantibodies in a crossmatch procedure. With AET (2-aminoethylisothiouronium bromide)-treated ^{51}Cr tagged platelets as targets, they were able to detect platelet antibodies in all nine sera from refractory patients. A critical evaluation of this method for crossmatching requires the inclusion of sera from non-refractory patients during platelet support and a direct comparison with the *in vivo* transfusion effect. This has not been done so far. We have evaluated this assay in a crossmatch procedure with sera from both refractory and nonrefractory patients. To account for spontaneous lysis, a standard serum panel was systematically included in all tests. Serum samples obtained at 2 weeks post-transfusion were also tested in the crossmatch procedure. All crossmatch tests were performed after the platelet transfusions and analysed in retrospect.

MATERIALS AND METHODS

Sera. Serum samples were obtained from patients with acute leukaemia receiving random single donor platelets for thrombocytopenia due to bone marrow failure (platelet count below 20×10^9/l). At the time of study, none had splenomegaly, fever $\geq 38{\cdot}5$°C, septicaemia or disseminated intravascular coagulation.

Sera were prepared from whole blood allowed to clot for 2 h at room temperature, inactivated for 30 min at 56°C and stored at −70°C. Two serum samples were obtained from each patient. The first one was taken immediately prior to the relevant platelet transfusion, and the second, 2 weeks after the platelet transfusion. For direct comparison, both serum samples were tested in the same experiment. Sera from multiply transfused patients with polyspecific platelet alloantibodies served as positive controls. Negative control sera were obtained form untransfused random male donors (laboratory personnel) of blood group O ($n=8$) and blood group A ($n=2$); ABO incompatibility between the control serum and the target platelets did not lead to platelet lysis (unpublished results).

Preparation of platelet suspensions. For crossmatch testing, target platelets were obtained from donors whose platelets were used for transfusion. 10 ml of freshly drawn EDTA blood (Venoject system, Terumo) was centrifuged at 300 *g* for 10 min. The platelet rich plasma (PRP) was concentrated at 1100 *g* for 15 min. The residual platelet poor plasma (PPP) was heat inactivated (30 min, 56°C) and centrifuged for 10 min at 300 *g*. PPP was diluted (1:5) with 0·013 M phosphate buffered 0·145 M NaCl, pH 7·4 (PBS). A volume of 0·7 ml of PPP/PBS was used for resuspension of the platelet pellet and 100 μCi ^{51}Cr (sodium chromate, Amersham International, Amersham, U.K.) in 0·1 ml 0·145 M NaCl was added. Following incubation for 45 min at room temperature, the platelets were washed twice with 2 ml PPP/PBS (7 min; 1700 *g*) and the final platelet concentration was adjusted to 1×10^8/ml with PPP/PBS.

^{51}Cr platelet lysis test. The test procedure was derived from Aster & Enright (1969) with some modifications. 0·02 ml of ^{51}Cr tagged donor platelets, 0·02 ml of serum, 0·02 ml of 0·05 M $MgCl_2$ in 0·145 M NaCl and 0·04 ml of freshly frozen rabbit serum, as a constant source of complement, were incubated in the wells of a microtitre plate (U shaped; Microtiter System, Greiner, West Germany) for 2·5 h at 37°C. The rabbit serum showed strong complement

activity and gave no toxicity when tested in the NIH lymphocytotoxicity test. The same batch of complement was used throughout the tests. After incubation, the plates were centrifuged at 750 *g* for 30 min. Half of the supernatant (0·05 ml) was taken off for counting the radioactivity in a gamma counter (Searle, model 1195, Nuclear Chicago Division). Consequently, for use in calculations, the counts of the supernatant were multiplied by a factor of 2. All tests were run in triplicate.

Results were expressed as the Z-score* (difference between test values and controls relative to the standard deviation of control values) and percentage relative counts† (percentage of counts released in supernatant of patient serum compared with total counts added). In addition, the values for Z-score and percentage relative counts were considered in combination.

The percentage relative counts induced by control sera exceeded 13% in only one experiment in which the percentage relative counts of all 10 control sera exceeded 13%. This test has therefore been excluded form further evaluation. In all other tests neither the mean value of 10 control sera nor that of the individual sera ever exceeded 13% relative counts.

Platelet transfusions. Patients received exclusively random single donor platelet transfusions from the start of platelet therapy. Platelets were obtained from random single donors by continuous flow centrifugation (Celltrifuge I, Aminco) according to previously described procedures (Sintnicolaas *et al.* 1981). A standardized dose of platelets (4×10^{11}) was administered per transfusion. 28 transfusions were studied in 14 patients. Just prior to and at 1 h following transfusion, a blood platelet count was done in triplicate (E.L.T.-8, Ortho Instruments, Westwood, Mass., U.S.A.) and the *in vivo* recovery of the platelets was calculated by use of the following formula:

$$\text{Recovery (\%)} = 100 \times \frac{\text{Platelet increment} \times \text{Blood volume}}{\text{No. of platelets transfused}}$$

Blood volume (l) was estimated to be 2·5 times body surface area (m^2) (Brassine, 1968).

Definition of successful versus non-successful platelet transfusions. Platelet recoveries of less than 20% at 1 h post-transfusion were considered failures. We used the 1 h post-transfusion platelet recovery (PTPR) as this may be a more reliable indicator of platelet alloimmunization than the 24 h PTPR (Daly *et al.* 1980). According to this criterion, 21 transfusions were successful and seven unsuccessful.

RESULTS

The results of the ^{51}Cr lysis tests were calculated as relative counts, Z-score or as the combination of these. The frequencies of false positive and false negative test results obtained using either method were not statistically different (Table I). Moreover, a comparison of Z-

* Z-score $= (p - x)/SD$; p refers to mean counts in supernatant of patient serum; x refers to mean counts of supernatants of 10 simultaneously run control sera, SD refers to standard deviation of x.

† Percentage relative counts $= (p/t) \times 100\%$; t refers to counts of 0·02 ml platelet suspension (pipetted separately into counting tubes).

Table 1. Frequency of false positive and false negative results in the ^{51}Cr platelet lysis assay using different criterion variables at a given cut-off point

Criterion (cut-off point)	Frequency of false positive test results	Frequency of false negative test results
Relative counts (13%)	1/7 (14%)	1/21 (5%)
Z-score (3)	2/8 (25%)	1/20 (5%)
Relative counts (16%) + Z-score (16)	1/7 (14%)	1/21 (5%)

score and percentage relative counts showed a positive linear correlation ($r = 0{\cdot}94$; Fig 1). Isotope release, expressed as percentage relative counts and Z-score, was related to the clinical transfusion recoveries at 1 h post-transfusion (Fig 1). No correlation was observed between the percentage of relative counts and the 1 h post-transfusion platelet recovery (Fig 2). Results of the tests with the 2-week post-transfusion sera were compared with those of the crossmatch in individual patients. Test results were calculated by using the percentage relative counts as the criterion variable. All patients with a positive crossmatch were also positive in the test with the 2-week post-transfusion serum. Of note is that the sera from four of 14 patients were initially negative, but became positive after the transfusion.

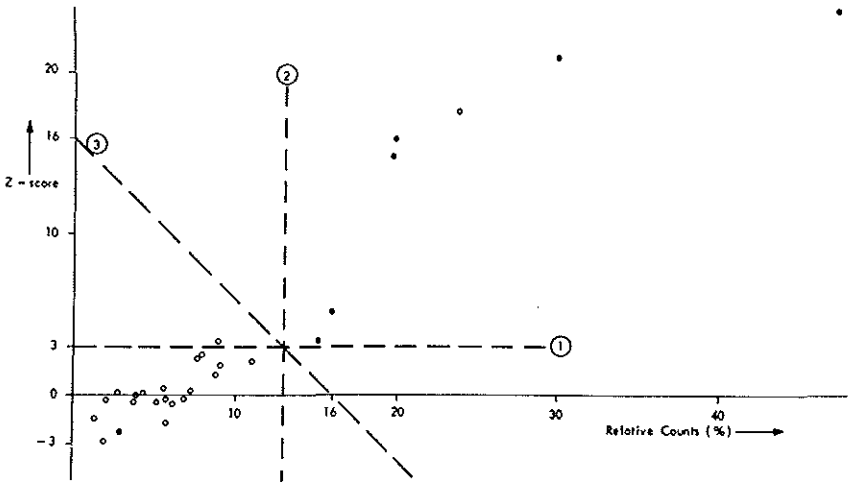

Fig 1. Relationship between results of platelet transfusion and two criterion variables of the ^{51}Cr platelet lysis assay. Closed circles indicate unsuccessful transfusions, open circles successful transfusions. Interrupted lines indicate: (1) cut-off point of Z-score; (2) cut-off point of relative counts; (3) cut-off line of combination Z-score and relative counts.

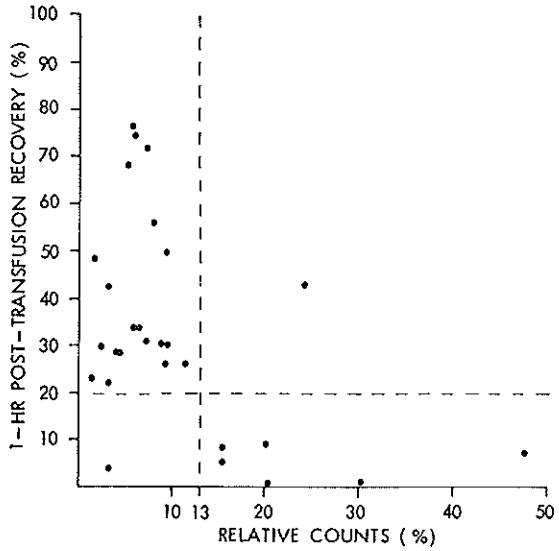

Fig 2. Relationship between 1 h post-transfusion platelet recovery and percentage relative counts of the ^{51}Cr platelet lysis assay.

DISCUSSION

Spontaneous lysis may be a serious problem with the ^{51}Cr platelet lysis assay (Aster & Enright, 1969; Cimo & Gerber, 1979). In our test procedure we have given special attention to this problem using 10 control sera and by comparing different methods to calculate the test results. It appeared that using the percentage relative counts, the test required only one or two control sera, provided that spontaneous lysis of control serum did not exceed 13%.

Using a 1 h post-transfusion platelet recovery of 20% as the lower limit of a successful transfusion, false negative results were seen in one of 21 and false positive results in one of seven cases. This indicates that this test may be a useful predictor of platelet transfusion results. These overall results are comparable to those of other tests, i.e. platelet immunofluorescence (Myers *et al*, 1981), a radiolabelled antiglobulin test (Kickler *et al*, 1983), and a mixed passive haemagglutination test (Shibata *et al*, 1981). This does not necessarily mean that these tests have identical predictive values in individual patients. To establish the proper role for each test for prospective platelet selection further comparative studies are needed, not only paying attention to the quality of the test results but also to the feasibility of the tests, as all tests are laborious. The ^{51}Cr-lysis assay offers the possibility to screen donors for alloimmunized patients; a disadvantage may be that fresh test platelets are required.

The 2-week post-transfusion serum was tested against the original platelet donor and showed conversion from negative to positive in four of 14 patients. Apparently, immunization (against the previous donor) sometimes occurred after a single transfusion. This suggests

that the continued employment of platelets from the same donor would have produced insufficient transfusion results at that time, but this was not verified. Subsequent platelet transfusions from other random single donors were, at least initially, not affected. The clinical relevance of this immunization for long-term transfusion support is currently under investigation, since three of the four patients with a seroconversion later developed broad alloimmunization. The other 10 patients with a single negative crossmatch and without a seroconversion did not show evidence of later alloimmunization.

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CHAPTER 5

A MICROPLATE ELISA FOR THE DETECTION OF PLATELET ALLOANTIBODIES; COMPARISON WITH THE PLATELET IMMUNOFLUORESCENCE TEST

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A microplate ELISA for the detection of platelet alloantibodies: comparison with the platelet immunofluorescence test

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Summary. An enzyme-linked immunoassay (ELISA) for the detection of platelet alloantibodies has been compared in detail with the platelet immunofluorescence test (PIFT). The ELISA appeared much simpler to perform than the PIFT. Both tests were comparable with regard to reproducibility and sensitivity. Alloantibodies were detected with ELISA and PIFT in 13 out of 14 patients who were refractory to random donor platelets. The value of these tests as a platelet crossmatch assay was determined in a retrospective comparison of the test results and the clinical transfusion responses expressed as the 1 h post-transfusion platelet recovery. 39/41 (95%) negative ELISA crossmatches and 30/33 (91%) negative PIFT crossmatches appeared to be associated with a successful platelet transfusion, whereas 7/10 positive ELISA crossmatches and 2/4 positive PIFT crossmatches appeared to be associated with transfusion failures. The high frequency of 'correct' negative tests indicates the importance of both assays in the prospective selection of compatible platelet donors for alloimmunized patients. However, because of its simplicity, the ELISA appears the method of choice for this purpose.

The detection of platelet alloantibodies is of major importance in platelet transfusion therapy. Alloimmunization to random donor platelets occurs frequently in patients on platelet support, resulting in the absence of a platelet transfusion response (Tejada *et al*, 1973; Dutcher *et al*, 1981). Further effective platelet support is then dependent on the availability of antigenically compatible platelet donors. *In vitro* tests to detect platelet alloantibodies are thus essential in establishing alloimmunity as the cause of platelet refractoriness, especially in the presence of other factors causing decreased platelet recoveries (e.g. fever, septicaemia, splenomegaly and disseminated intravascular coagulation). Moreover, these assays should allow compatibility testing of platelet donors derived from an HLA-typed donor pool or even from a random donor population. To fulfil these criteria, tests for detection of anti-platelet antibodies should be reliable, sensitive, rapid, and allow the use of preserved donor cells. Khan *et al* (1981) reviewed a variety of techniques described for this purpose. The present serological techniques focus on the demonstration of platelet-binding immunoglobulins (PBIg). We have developed an enzyme-linked immunosorbent assay (ELISA) which is based on that of Horal *et al* (1981). In the ELISA, platelets are coated onto the bottom of a microtitre plate and used as a solid phase. The performance of the ELISA was compared with the immunofluorescence test on platelets in suspension (PIFT) (von dem Borne *et al*, 1978), which is a sensitive assay to identify platelet alloantibodies (Pegels *et al*, 1982), and which has been shown to offer additional value over HLA typing in platelet crossmatching (Brand *et al*, 1978). The PIFT, however, has some disadvantages in that it is time consuming and, although allowing visual inspection of the platelet–antibody reaction, is subject to observer bias. We have demonstrated that the reproducibility, sensitivity and platelet crossmatch results are comparable for ELISA and PIFT. The ELISA, however, is much faster, is objective, and thus meets the criteria set out above.

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MATERIALS AND METHODS

Preparation of platelet suspensions. Blood was drawn into EDTA (Venoject System, Terumo). Platelet-rich plasma (PRP) was obtained by centrifuging at 400 *g* for 10 min. All centrifugation steps were carried out at 20°C using a temperature-controlled centrifuge. The PRP was centrifuged at 1200 *g* for 20 min to obtain a platelet pellet. For use in the

ELISA, platelets were washed four times in a solution of 0·009 M Na_2EDTA in 0.013 M phosphate-buffered 0·145 M NaCl, pH 7·4 (EDTA–PBS) and finally resuspended in EDTA–PBS (100×10^9/l). Platelet counts were measured by a laser haematology counter (ELT-8, Ortho Instruments). For use in the PIFT, the platelet pellet was washed three times with EDTA–PBS, followed by a fixation step with paraformaldehyde (von dem Borne *et al*, 1978). The fixed platelets were washed twice in EDTA–PBS and finally resuspended in EDTA–PBS (200×10^9/l).

Platelet ELISA. 50 µl of the platelet suspension (5×10^6 platelets) were placed in the wells of a microtitre plate (Greiner, cat. no. 655101). The plate was centrifuged for 5 min at 300 *g* at room temperature (RT) and placed in an incubator for 15 min at 37°C. The plate was washed four times with PBS-Tween (0·05% v/v), by filling the wells with the wash-solution and flicking the plate over a sink. Tween was added to the wash solution since it reduces the background caused by non-specific binding of serum-Ig to the plastic. Incubation with bovine serum albumin did not further reduce this background and was therefore not done. 50 µl of undiluted serum were added and incubated for 60 min at 37°C. After four wash cycles, 100 µl of $F(ab')_2$-fragments of goat–anti-human IgG conjugated to horseradish peroxidase (Cappel, Cooper Biomedical), diluted 1 in 10 000 in PBS–Tween–BSA (4%) were added and incubated for 30 min at 37°C. After washing four times, 100 µl of a freshly prepared substrate solution (80 mg of orthophenylenediamine in 50 ml citrate/phosphate buffer 0·025 M/0·04 M, pH 5·0, to which 20 µl 30% H_2O_2 is added just before use) was added and the plates were placed at RT in the dark. After 10 min, the enzyme reaction was stopped by adding 50 µl 4 N H_2SO_4. Optical densities (O.D.) of the wells were read by an automated micro-ELISA-reader (Titertek, Multiscan MC) at 492 nm (against 620 nm, dual wave length).

Calculation of results. All tests were performed in triplicate and mean values were used for further calculations. The platelets from a single donor were coated onto the wells of individual plates, and 10 negative control and one positive control sera were run in parallel. The test result was expressed as the ratio of the O.D. value of a test serum to the mean of the O.D. values of 10 negative control sera. A ratio of 1·5 or more was considered to be positive. Calculations were performed using a microcomputer on-line with the ELISA-reader.

Platelet immunofluorescence test (PIFT). The PIFT was performed according to the method of von dem Borne *et al* (1978). All tests were performed in duplicate and all slides were independently read by two observers. Fluorescence intensity was scored from −, +, ++, +++, to ++++.

Patient/control sera. Serum samples were harvested from whole blood which had been allowed to clot for 2 h at RT, and stored at −70°C. Sera from multitransfused patients reacting with platelets and lymphocytes from a high percentage of random donors (MS sera) and an anti-Zw^a (anti-PI^{a1}) containing serum (gift from Dr A. E. G. Kr. von dem Borne, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) served as positive controls. Negative control sera were obtained from untransfused male blood donors. Control and test sera were always ABO compatible with the test platelets unless otherwise stated.

Platelet crossmatching. To determine the value of ELISA and PIFT for platelet crossmatching, a retrospective analysis was made using the method described by Van der Velden *et al* (1986). Patients with acute leukaemia receiving exclusively random single donor platelets from the start of platelet therapy were studied. Platelets for transfusion were obtained by continuous flow centrifugation (Celltrifuge I, Aminco) according to the procedure described by Sintnicolaas *et al* (1981). Just prior to and at 1 h following transfusion, a blood platelet count was done in triplicate (ELT-8), and the *in vivo* recovery of the platelets was calculated by use of the following formula:

$$\text{recovery (\%)} = 100 \times \frac{\text{platelet increment} \times \text{blood volume}}{\text{number of platelets transfused}}$$

Blood volume (litres) was estimated to be 2·5 times body surface area (m^2) (Brassine, 1968). Platelet recoveries of less than 20% at 1 h post-transfusion were considered as failures. We used the 1 h post-transfusion platelet recovery as this appears to be a more reliable indicator of platelet alloimmunization than the 24 h post-transfusion platelet recovery (Daly *et al*, 1980). Platelet crossmatches were performed in both assays using test platelets from the donor of the platelets used for the transfusion, and patient serum obtained just before (within 12 h) the platelet transfusion.

RESULTS

Reproducibility of ELISA and PIFT; correlation of results between the two assays

Sera from 40 patients with acute leukaemia were tested on three different days against platelets derived from two volunteers using ELISA and PIFT simultaneously. In Fig 1 the day-to-day variation in the results of the ELISA test for the 80 cell–serum pairs is shown. The ELISA showed reproducible results on the three occasions in 70/80 (88%) cell–serum pairs tested (Table I). From Fig 1 it is clear, however, as expected, that the reproducibility of the ELISA in terms of being positive or negative depends upon the level of reactivity of the cell–serum pairs. Tests with a high ratio ($\geq 2{\cdot}0$, test value over control value) were always positive on repetition, whereas tests with a low ratio ($\leq 1{\cdot}1$) were repeatedly negative. Discrepant results were obtained only when the test ratio was near the cut-off level (1·5). The average paired Spearman rank correlation between the ELISA 3 d repeated reproducibility tests was $r_s = 0{\cdot}95$. The PIFT, like the ELISA, also showed reproducible results in 70/80 (88%) cell–serum pairs tested (Table I). The average paired Spearman rank correlation between the PIFT 3 d repeated reproducibility tests was $r_s = 0{\cdot}90$. A strongly positive PIFT (+++ or ++++) was never negative when repeated.

We have compared the 240 ELISA ratios of the 80 cell serum pairs tested on three separate days with the corresponding 240 PIFT scores (Fig 2). The average Spearman rank correlation between PIFT and ELISA was $r_s = 0{\cdot}80$. A positive (or conversely negative) ELISA with a negative (conversely positive) PIFT was found in 21/240 = 9% (12/240 = 5%) of

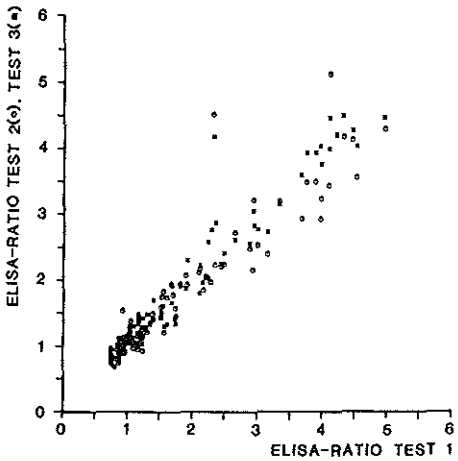

Fig 1. Reproducibility of the platelet-ELISA; 40 patient sera were tested against platelets from two donors on three different days. The horizontal axis indicates the results from test 1, the vertical axis represents the results from test 2 (o) and test 3 (*).

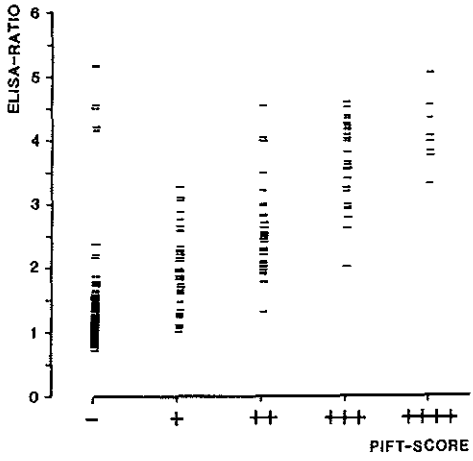

Fig 2. Comparison of the test results of platelet-ELISA and PIFT; scattergram of 240 observations obtained from 80 cell–serum pairs tested on 3 d in the reproducibility experiment.

Table I. Reproducibility of ELISA and PIFT

Test no.*		
1 2 3	ELISA	PIFT
+ + +	36	32
– – –	34	38
+ + –	3	4
+ – –	7	6

+: positive test result; –: negative test result.

* Three independent experiments.

Table II. Comparison of sensitivity of ELISA and PIFT

Serum tested	Cell donor 1		Cell donor 2	
	ELISA	PIFT	ELISA	PIFT
A	256	256	1	4
B	1	2	16	4
C	8	8	16	32
D	1	—	16	16
E	2	2	2	1
F	32	16	16	16
G	32	128	4	2
H	4	4	—	—
I	8	8	8	8

A–H: MS-sera; I: anti-Zw^a serum. Figures indicate reciprocal of titre.

the cases. The five tests with an ELISA ratio > 4 but negative in PIFT involved a serum sample from the same individual.

Influence of ABO-incompatibility between platelets and serum on the ELISA

Platelets from five donors with blood group A were tested in the ELISA against 10 negative group O control sera. Six sera reacted with all donor cells, one serum with four out of five donor cells, and three sera were negative. Test ratios of positive reactions ranged from 1·7 to 3·7. This indicated that anti-A antibodies were detected in most sera, and that in testing for platelet alloantibodies ABO incompatibility between test platelet and test serum must be avoided.

Comparison of sensitivities of ELISA and PIFT

Serial dilutions of eight MS-sera and one anti-Zw^a serum were tested against the platelets of two individuals. Sera were diluted in pooled negative control sera. Titres were identical for ELISA and PIFT on nine occasions and differences between ELISA and PIFT scores never exceeded two doubling dilutions. One cell–serum pair was negative in the PIFT but had a titre of 1 in ELISA (Table II).

Detection of platelet alloantibodies in patient sera

Sera from 14 patients suffering from acute leukaemia were

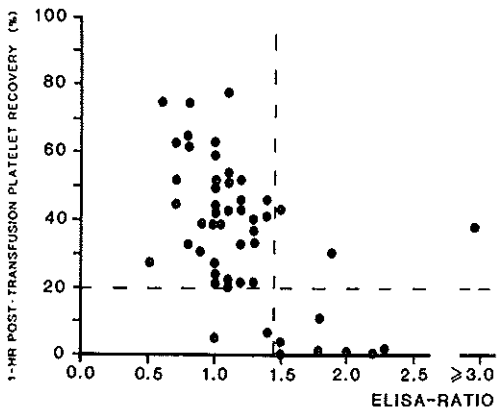

Fig 3. Platelet crossmatches. Relationships between the 1 h post-transfusion platelet recovery and the ELISA ratio. The horizontal interrupted line indicates the cut-off level for a successful/unsuccessful platelet transfusion; the vertical interrupted line indicates the cut-off level for a negative/positive ELISA.

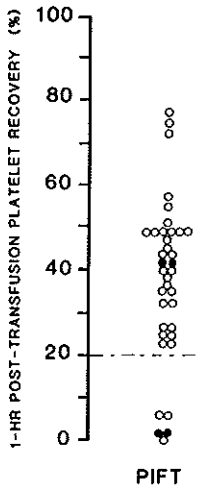

Fig 4. Platelet crossmatches. Relationship between the 1 h post-transfusion platelet recovery and the result of the PIFT. Open circles indicate a negative PIFT, closed circles indicate a positive PIFT. The horizontal interrupted line indicates the cut-off level for a successful/unsuccessful platelet transfusion.

tested against a panel of platelets from 10 random donors using both ELISA and PIFT. All patients tested were refractory to random donor platelets, i.e. the 1 h post-transfusion recovery was below 20% on two successive occasions in the absence of non-immune factors known to interfere with platelet recovery. Antibodies against platelets were detected in the sera from 13 patients in ELISA and PIFT. The serum derived from one patient was negative in both assays.

Platelet crossmatches

Platelet crossmatches were performed using the ELISA on 51 occasions and using the PIFT on 37 occasions. A clear correlation between the value of the ELISA-ratio and the 1 h post-transfusion platelet recovery was found (Fig 3). The frequency of false positive tests (i.e. a 1 h post-transfusion platelet recovery above 20% in combination with a positive test result) was low in both the ELISA and PIFT: 3/42 and 2/32 respectively (Figs 3 and 4). False negative results (i.e. a 1 h post-transfusion platelet recovery below 20% associated with a negative test result) were more frequently observed, occurrring in 2/9 instances for the ELISA and in 2/5 instances for the PIFT. Negative crossmatches correlated with successful platelet transfusion in the ELISA on 39/41 occasions and in the PIFT on 30/33 occasions. Positive tests correlated with transfusion failure on 7/10 and 2/4 occasions respectively. Thus, the predictive value of a negative test for a successful transfusion was 95% for the ELISA and 91% for the PIFT.

DISCUSSION

The reproducibility of the platelet ELISA appeared to be satisfactory and was comparable to that of the PIFT. Also, as expected, reproducibility was dependent on the strength of the reaction of the serum with the cells. Therefore, a comparison of experiments reported in various publications would be possible only if the level of reactivity of the sera tested was known, e.g. negative controls in combination with strongly reactive sera result in an overestimation of the reproducibility. No details are given as to the level of reactivity of test sera in the literature previously described ELISAs (Schiffer & Young, 1983; Taaning, 1985), and hence a meaningful comparison with our ELISA cannot be made.

A clear correlation is found between the value of the ELISA ratio and the PIFT score. This is not surprising as both assays are based on the detection of Ig bound to platelets. However, one serum was strongly positive in the ELISA but negative in

the PIFT. Since the patient from whom the serum was obtained was not refractory to random donor platelets, we conclude that the ELISA yielded a false positive result. The reason for this is not known, but it was not due to increased non-specific binding of serum Ig to the plastic (data not shown). In the ELISA, ABO incompatibility between platelets and negative control serum frequently resulted in positive results, as was the case for the PIFT (data not shown), confirming earlier data (von dem Borne *et al*, 1978). This may be explained by the specific binding of anti-A antibodies in selected group O sera to platelets expressing the A antigen (Kelton *et al*, 1982).

The sensitivities of the ELISA and PIFT were comparable, as determined in dilution experiments and in screening sera from patients who were refractory to random donor platelets. Thus, both tests are equally suitable for the identification of antibodies reactive with donor platelets. Successful platelet transfusion in alloimmunized patients calls for a reliable platelet crossmatch test. The ELISA is shown in this retrospective study to have a high (95%) predictive value for a successful platelet transfusion, and may thus be instrumental in selecting compatible platelet donors for alloimmunized patients. Moreover, the frequency of a positive ELISA result correlating with a successful transfusion (3/10 cases) was rather low. Hence, the number of donors unnecessarily deferred will also be low.

Our data are comparable with those obtained in other platelet crossmatch tests, e.g. a ^{51}Cr-lysis test (Van der Velden *et al*, 1986), a radioactive antiglobulin test (Kickler *et al*, 1984; Freedman *et al*, 1984) and a radiolabelled protein-A and a peroxidase anti-peroxidase test (Yam *et al*, 1984). However, the advantages of the ELISA over these assays are: (a) no radioactive waste; (b) test results are objective; (c) platelets can be prepared and stored in microtitre plates (Schiffer & Young, 1983; Taaning, 1985), and (d) only the ELISA allows rapid screening of many donors against one patient. Therefore, the ELISA is the method of choice for further studies on the selection of compatible platelet donors for alloimmunized patients. Of course, prospective studies are warranted. Preliminary data (not shown) confirm the value of the ELISA for donor selection.

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CHAPTER 6

SIMULTANEOUS FLOW CYTOMETRIC DETECTION OF ANTIBODIES AGAINST PLATELETS, GRANULOCYTES AND LYMPHOCYTES

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Simultaneous flow cytometric detection of antibodies against platelets, granulocytes and lymphocytes

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We present a time-saving and objective flow cytometric immunofluorescence assay for the simultaneous detection of antibodies against platelets, granulocytes or lymphocytes using a reconstituted mixture of these cell populations. Platelets, granulocytes and lymphocytes could be distinguished on the basis of their forward (FSC) and sideways (SSC) light scattering properties plotted on scales of 4 log orders. After setting FSC/SSC gates around the platelets, granulocytes and lymphocytes, the reactivity of the sera with the cell populations was determined by histogram analyses of immunofluorescence for each gate. The flow cytometric assay of reconstituted cell mixtures showed a strong, positive correlation with a reference microscopic immunofluorescence assay of separate cell suspensions. The reproducible procedures for the isolation and staining of the cells and the electronic stability of the flow cytometer permitted the use of the same gate and marker settings throughout the experiments. Consequently, the entire analysis of data stored in list mode could be performed using a keystroke, so that time consuming and subjective manual analyses were avoided.

Key words: Flow cytometry; Platelet; Granulocyte; Lymphocyte; Immunofluorescence; (Antibody)

Introduction

Antibodies against platelets, granulocytes and/or lymphocytes are important in immunohematology, e.g., in autoimmune hematological disease and in platelet transfusion therapy. The demonstration of autoantibodies on platelets and/or granulocytes may be valuable in establishing the autoimmune nature of thrombocytopenia or granulocytopenia (Verheugt et al., 1977; Von dem Borne et al., 1980). In platelet transfusion therapy, serological tests for the demonstration of alloantibodies against HLA antigens and/or platelet-specific antigens may establish alloimmunization as the cause of platelet refractoriness; such tests may also be used as a platelet crossmatch to select compatible platelet donors for alloimmunized patients (Freedman et al., 1984; Sintnicolaas et al., 1987).

Direct and indirect immunofluorescence techniques allow the detection of autoantibodies and/or alloantibodies to platelets, granulocytes

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Abbreviations: BSA, bovine serum albumin; FSC, forward light scatter; GIFT, granulocyte immunofluorescence test; LIFT, lymphocyte immunofluorescence test; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PIFT, platelet immunofluorescence test; PRP, platelet rich plasma; RT, room temperature; SSC, sideways light scatter.

or lymphocytes. The assays are routinely performed on suspensions of blood cells containing single, i.e., separated, purified cell populations (platelets, granulocytes or lymphocytes). Flow cytometry enables these cell populations to be distinguished in a mixed suspension on the basis of their different light scattering properties. The application of flow cytometry to the detection of antibodies against platelets, granulocytes and lymphocytes would permit the simultaneous detection of antibodies against those cells after electronic gate setting. We present here a simple and rapid immunofluorescence assay for the detection of autoantibodies and alloantibodies to platelets, granulocytes and lymphocytes that is performed using a reconstituted cell suspension and flow cytometric analysis.

Materials and methods

Preparation of cell suspensions

For screening sera for the presence of antibodies against platelets, granulocytes or lymphocytes, a cell panel derived from five randomly chosen donors was routinely used. Platelet-rich plasma (PRP) was obtained from EDTA-anticoagulated blood by centrifuging at $400 \times g$ for 10 min. All centrifugation steps were carried out at 20 °C using a temperature controlled centrifuge (Rotixa RP, Hettich, F.R.G.). The PRP was centrifuged at $1200 \times g$ for 20 min to obtain a platelet pellet. The platelets were washed twice in a solution of 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). To prepare granulocyte and mononuclear cell suspensions, EDTA-PBS-BSA was added to the 4–6 ml of cell suspension that remained after removal of PRP to give a final volume of 10 ml. To this suspension, 0.5 ml of methylcellulose solution (2% in PBS), was added, mixed and incubated at 37 °C for 30–45 min to allow gravity sedimentation. The leukocyte containing supernatant was harvested and the granulocytes were separated from the mononuclear cells by density centrifugation using Ficoll-Hypaque (Lymphoprep, Nycomed, Norway) at $1000 \times g$ for 20 min. The interphase cells (mononuclear cells) were collected and washed once with EDTA-PBS-BSA. The erythrocytes in the cell pellet fraction were lysed by incubation with precooled NH_4Cl for 5 min on melting ice. The remaining cells (granulocytes) were washed once with EDTA-PBS-BSA. The three fractions were then washed once with EDTA-PBS without BSA, fixed with 1% paraformaldehyde (PFA) in PBS for 5 min at room temperature (RT) and washed twice using EDTA-PBS-BSA. After resuspension in EDTA-PBS-BSA, the cell concentrations were adjusted to 150×10^9/l for platelets and 4.5×10^9/l for mononuclear cells and granulocytes. Finally, a mixed cell suspension was prepared by mixing equal volumes of the platelet, granulocyte and mononuclear cell suspensions.

Preparation of 1% PFA in PBS

A stock solution of 4% (w/v) PFA was prepared by dissolving PFA in PBS while slowly heating to 70 °C. The solution was filtered and stored in the dark at 4 °C. The stock solution was used for a maximum of 4 weeks. A working solution of 1% PFA was prepared by dilution of the stock solution with PBS immediately before use.

Indirect immunofluorescence test

To 50 µl of the mixed cell suspension 50 µl of serum were added. Following incubation for 30 min at RT the cells were washed three times. 50 µl of a 1/40 dilution of goat $F(ab')_2$-anti-human IgG conjugated with fluorescein isothiocyanate (FITC) (Dakopatts, Glostrup, Denmark) were added for 30 min at RT, followed by washing and flow cytometry. All tests were performed in duplicate.

Flow cytometry

Flow cytometry was performed on a FACScan (Becton and Dickinson Immunocytometry Systems (BDIS), San Jose, CA), equipped with an air-cooled argon ion laser emitting 488 nm at 15 mW, a solid state silicon detector to detect forward light scatter (FSC) and three photomultiplier tubes to detect sideways light scatter (SSC), and green (FITC), orange-red and deep red fluorescence. Green fluorescence was collected through a 530/30 nm bandpass filter. The FSC,

SSC and FITC fluorescence signals were amplified logarithmically. The data of 10,000 events were collected, stored in list mode and analyzed using Consort 30 software.

In the analyses, gates were set around the platelets, granulocytes and lymphocytes on the basis of their FSC and SSC characteristics. Histogram analyses of FITC fluorescence were performed for these 3 gates in which standard markers were placed to distinguish negative and positive events. These standard marker sets were chosen as follows. One positive and one negative serum was selected on the basis of their reactivity in the microscopic assay. These sera were then tested against platelets, granulocytes and lymphocytes from 20 donors in the flow cytometric and microscopic assays. For each gate, five different marker sets were arbitrarily chosen around the feet of the negative peaks obtained with the negative serum, and the percentages of positive cells calculated. These results were compared with those of the microscopic assays and the marker settings that gave maximum discrimination between microscopic negative and positive results were selected: PIFT, channels 200 and 10,000; GIFT, channels 460 and 10,000; and LIFT, channels 340 and 10,000. The standardized isolation and staining procedures of the cells permitted the use of identical gate and marker settings throughout the experiments. All tests were performed in duplicate and the mean values were used as the test results. A test result was scored 'positive' when the percentage of positive cells exceeded 30%. That percentage resulted in the best positive correlation between the flow cytometric and microscopic results of PIFT, GIFT and LIFT assay (data not shown). A test result was rejected when the difference between the duplicate values exceeded 10%.

Microscopic immunofluorescence tests

The platelet immunofluorescence test (PIFT), granulocyte immunofluorescence test (GIFT) and lymphocyte immunofluorescence test (LIFT) were performed as previously described (Decary et al., 1975; Verheugt et al., 1977; Von dem Borne et al., 1978). Cell suspensions of platelets, granulocytes and mononuclear cells were prepared as described above. Final cell concentrations were 100×10^9/l for platelets and 1.5×10^9/l for granulocytes and mononuclear cells. All tests were performed in duplicate and all slides were independently read by two investigators. Fluorescence intensity was scored from negative to strongly positive, i.e., −, +, + + to + + +.

Patient and control sera

Serum samples were harvested from whole blood which had been allowed to clot for 2 h at RT and stored at −70 °C. Sera from multitransfused patients reacting with platelets, granulocytes and lymphocytes from more than 90% of random donors (MS sera) served as positive controls. Negative control sera were obtained from untransfused male blood donors. Control and test sera were always ABO compatible with the test cells.

Monoclonal antibodies

For the identification of lymphocytes we used anti-Leu4/FITC (CD3, T lymphocytes) and anti-Leu16/FITC (CD20, B lymphocytes) (BDIS). Granulocytes were identified using VIM-D5 (CD15), monocytes using My-4 (CD14) (Coulter Clone, Hialeah, FL), platelets using CLB-thromb/1 (CD61) (Central Laboratory of the Dutch Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), and erythrocytes using VIE-G4 (reactive with glycophorin 'A). VIM-D5 and VIE-G4 were kindly donated by Dr. W. Knapp (Institute for Immunology, Vienna University, Vienna, Austria). When unconjugated monoclonal antibodies were used, a second incubation was performed with goat anti-mouse Ig-FITC (Nordic). All antibodies and conjugates were diluted in EDTA-PBS-BSA and used at saturating concentrations.

Results

The use of a 4-order logarithmic scale for FSC and SSC permitted the simultaneous display of platelets, granulocytes and lymphocytes. For each cell population gates were set as shown in Fig. 1. The cell mixtures were immunophenotyped to analyze the composition of the gated cell populations (Table I). In the platelet gate (gate 1), 79%

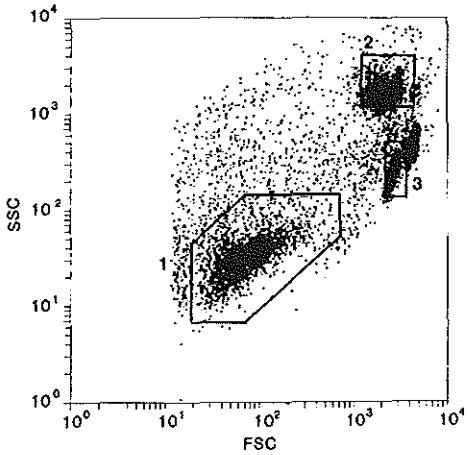

Fig. 1. FSC (horizontal axis)/SSC (vertical axis) dot plot of a reconstituted cell mixture. A 4 order logarithmic scale is used for both axes. The standard gates for platelets (gate 1), granulocytes (gate 2) and lymphocytes (gate 3) are shown. The cluster of events with FSC/SSC properties larger than lymphocytes represents the monocytes.

[3%] (mean [SD] of five experiments) of the cells were positive for CD61; in the granulocyte gate (gate 2), 80% [10%] of the cells were positive for CD15 and in the lymphocyte gate (gate 3), 84% [8%] of the cells were positive for either CD3 or CD20. The purity of the cell populations selected by these gates was considered sufficient to permit detection of antibodies against the respective cell populations.

A cell mixture prepared from a random donor was incubated with a serum containing multispecific anti-HLA antibodies and a negative control serum (Fig. 2). Using the gate settings as described above, separate FITC fluorescence histograms were obtained for platelets, granulocytes and lymphocytes. Clear differences between the fluorescence intensities of the negative and positive sera were observed in all three cases. The cells in the granulocyte and lymphocyte gates of the negative control sample revealed a second peak of cells with intermediate fluorescence. Additional experiments (not shown) revealed that these intermediate fluorescent cells also stained positive with propidium iodide. Thus, the intermediate fluorescence of the granulocytes may have been caused by cell membrane damage. Two colour immunofluorescence analysis of mononuclear cell suspensions (not shown) revealed that the cells with low fluorescence were mainly $CD3^+$ (i.e., T lymphocytes) and that the cells with intermediate fluorescence were mainly $CD20^+$ (i.e. lymphocytes) or $CD14^+$ (i.e., monocytes). Thus the intermediate fluorescence of the cells within the lymphocyte gate may result from (a) surface IgG on B lymphocytes and (b) aspecific-binding of serum IgG and/or conjugate to Fc receptors on monocytes present in the lymphocyte gate. The presence of cell populations with intermediate fluorescence led to an increase in the percentage of positive cells in the negative controls, but did not prohibit the detection of alloantibodies (see below).

To study whether or not the mixing procedure of the platelets, granulocytes and lymphocytes per se would influence the immunofluorescence signals, experiments were simultaneously performed using reconstituted cell mixtures and non-mixed cells, i.e., with separate suspensions of platelets, granulocytes and mononuclear cells

TABLE I

IMMUNOPHENOTYPING OF CELL POPULATIONS BY FLOW CYTOMETRY

Cell population	Phenotype	Platelet gate (#1)	Granulocyte gate (#2)	Lymphocte gate (#3)
Platelets	CD61	79 [3] [a]	4 [5]	2 [4]
Granulocytes	CD15	7 [3]	80 [10]	2 [1]
Monocytes	CD14	0 [1]	0 [0]	2 [2]
Lymphocytes	CD3 + CD20	2 [1]	6 [3]	84 [8]
Erythrocytes	Glycophorin A	0 [1]	2 [2]	0 [0]

[a] Mean [1 standard deviation] ($n = 5$).

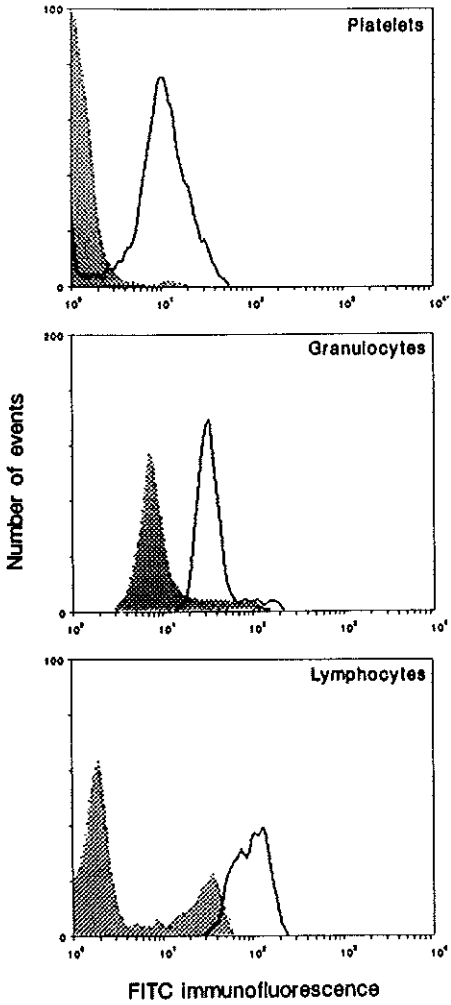

Fig. 2. Example of fluorescence histograms. On the horizontal axis FL1 (FITC fluorescence) is indicated on a log scale. On the vertical axis the number of events is given on a linear scale. Shaded curve: negative control serum. Open curve: positive control serum.

without further modification of the test. Three patient sera were selected that showed strong and selective reactivity against a single cell population (platelets or granulocytes or lymphocytes) in the conventional microscopic assay. These sera were tested in conjunction with a negative and a positive control serum against a cell panel derived from 5 randomly chosen blood group O donors. The results are shown in Fig. 3. For each serum and cell donor, the percentage of positive cells obtained using non-mixed cell supsensions was subtracted from the corresponding percentage obtained using reconstituted cell suspensions. The resulting differences (mean [SD]) were 4% [5%] ($p = 0.0003$; Wilcoxon signed rank test) for platelets, 2% [6%] ($p = 0.13$) for granulocytes and 0% [6%] ($p = 0.57$) for lymphocytes. The results obtained with mixed cells and separate cell suspensions were strongly and positively correlated (platelets, $r = 0.99$; granulocytes, $r = 0.98$; lymphocytes, $r = 0.99$). Thus, mixing of the cells did not significantly affect the immunofluorescence signals obtained from granulocytes and lymphocytes, and led to a small but significant increase in the percentage of positive platelets.

Finally, flow cytometric analyses were compared with microscopic analyses on separate cell suspensions for the detection of serum alloantibodies (Table II). 24 sera from patients with hematological malignancies receiving platelet transfusion therapy were tested in both assays against a panel derived from five randomly chosen blood group O donors. These experiments were performed on three occasions (eight sera and five cell donors per occasion). Flow cytometry and microscopy yielded identical scores for platelets in 108 of the 118 evaluable tests, granulocytes in 96 of 114 and lymphocytes in 100 of 117. An analysis of discrepant scores revealed that flow cytometry yielded significantly more positive reactions than microscopy for platelets and lymphocytes ($p = 0.002$ and $p < 0.001$ respectively). Thus, flow cytometry was more sensitive in the detection of antibodies against platelets and lymphocytes than microscopy. Flow cytometry and microscopy did not differ significantly in the detection of antibodies against granulocytes. In addition, the results were analyzed for each serum. A serum was scored reactive when cells of more than two out of five donors of the cell panel gave weakly positive reactions (microscopic score: +, flow cytometry 30–50% positive cells) or when cells of at least one donor of the panel gave

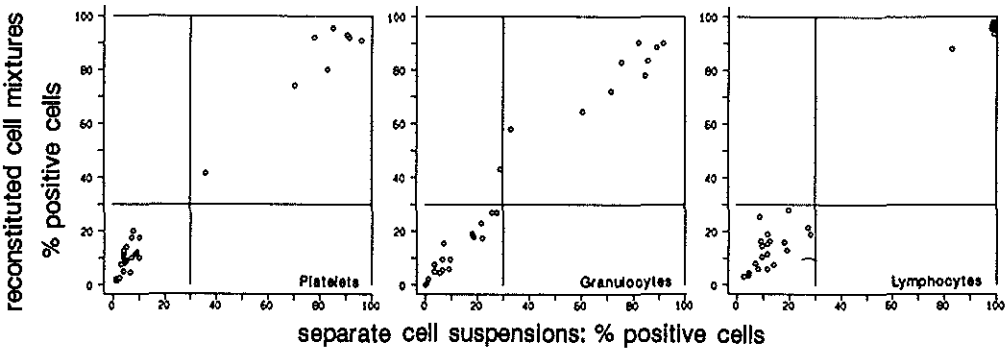

Fig. 3. Comparison of the use of reconstituted cell mixtures with separate cell suspensions. Horizontal axis: percentage of positive cells using separate cell suspensions. Vertical axis: percentage of positive cells using reconstituted cell mixtures. The 30% lines on both axes indicate the cut-off values for discrimination between negative and positive test results (see materials and methods section).

strongly positive reactions (microscopic score + + or + + +; flow cytometry assay > 50% positive cells). For 20 of the 24 sera tested, identical scores were obtained. No antibodies were detected in 14 sera, HLA antibodies (i.e., antibodies reactive with platelets, granulocytes and lymphocytes) were detected in four sera and platelet specific antibodies (i.e., antibodies reactive with platelets only) were demonstrated in two sera. Discrepant results were found in four sera: three were reactive only in the flow cytometric assay (two sera were reactive with lymphocytes only; one serum with granulocytes only) and one serum reacted in the flow cytometric assay with platelets and lymphocytes and in the microscopic assay with granulocytes and lymphocytes.

Discussion

Initially, immunofluorescence microscopic tests were performed on separate cell suspensions, i.e., containing only platelets, granulocytes or lymphocytes and thus requiring separate examinations (Decary et al., 1975; Verheugt et al., 1977; Von dem Borne et al., 1978). Apart from being time consuming, a major disadvantage of microscopic tests is that the results are subject to observer bias. Recently, flow cytometric tests have been described for the detection of alloantibodies against platelets (Rosenfeld et al., 1986; Wang et al., 1989) and lymphocytes (Garovoy et al., 1983; Scornik et al., 1989). Also, the simultaneous detection of alloantibodies against granulocytes,

TABLE II

COMPARISON OF THE FLOW CYTOMETRIC IMMUNOFLUORESCENCE ASSAY APPLIED TO RECONSTITUTED CELL MIXTURES WITH THE MICROSCOPIC METHOD APPLIED TO SEPARATE CELL SUSPENSIONS

Flow cytometric assay	Microscopic method	Platelets	Granulocytes	Lymphocytes
−	−	87	82	82
+	+	21	14	18
−	+	0 ($p = 0.002$ *)	10 (n.s.)	0 ($p < 0.001$)
+	−	10	8	17
Not evaluable		2	6	3

Key: − = negative test result; + = positive test result; * = sign test; n.s. = not significant.

lymphocytes and monocytes, but not platelets, has been reported (Robinson et al., 1987). The assessment of the reaction pattern of a serum against three blood cell populations of a single donor is important since information about the specificity of antibodies can thus be obtained. For instance, anti-HLA antibodies are reactive with platelets, granulocytes and lymphocytes, whereas antibodies against platelet or granulocyte specific antigens react only with platelets or granulocytes, respectively.

We present here a flow cytometric method for performing simultaneous immunofluorescence tests on platelets, granulocytes and lymphocytes from a single donor for the detection of antibodies against these cells. The use of 4-order logarithmic scales for FSC and SSC signals permitted the identification of platelets, granulocytes and lymphocytes by standard FSC/SSC gate settings in reconstituted cell mixtures prepared from separate cell suspensions. The cell mixtures were reconstituted in such a way that a similar number of events was recorded in the three gates. Contamination of these gated cell populations with other cell types was $< 20\%$ in most cases. A potentially time-saving alternative to the use of reconstituted cell mixtures would be the use of buffy coat preparations in which the erythrocytes have been lysed. However, the concentrations of platelets, granulocytes and mononuclear cells may differ considerably from donor to donor, leading to variable ratios between cell types on the one hand and antibodies on the other. Hence, we have not explored this approach further. To discriminate between positive and negative cells in each gate, markers were set on the basis of the fluorescence histograms of the negative control serum, which were kept constant throughout the experiments.

Using sera exclusively reactive with platelets, granulocytes or lymphocytes we demonstrated that the reconstitution procedure did not lead to major changes in the immunofluorescence signals compared to the separate cell suspensions. A slight but significant increase in the percentage of positive platelets was observed which did not interfere with the detection of antibodies to platelets. A comparison between the flow cytometric assay applied to reconstituted cell mixtures and the microscopic assay applied to separate cell suspensions revealed a greater sensitivity for the flow cytometric assay in detecting antibodies to platelets and lymphocytes, whereas both assays performed similarly in the detection of antibodies to granulocytes.

The standardized isolation and staining procedures of the cells and the electronic stability of the FACScan flow cytometer permitted unchanged gate and marker settings throughout the experiments. A major advantage of this situation was that the analysis and calculation of the results could be fully automated through a keystroke avoiding the time consuming and subjective adjustments of the gate and marker settings which would have required manual analysis.

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CHAPTER 7

DETECTION OF PLATELET ANTIBODIES BY SOLID PHASE RED CELL ADHERENCE TEST, PLATELET ELISA AND FLOW CYTOMETRIC IMMUNOFLUORESCENCE ASSAY: A COMPARITIVE STUDY

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ABSTRACT

Four different assays for the detection of platelet-reactive antibodies were compared in an in vitro study: a solid-phase red cell adherence assays (SPRCA), a solid-phase enzyme-linked immunoassay (ELISA) and a platelet and lymphocyte immunofluorescense test (PIFT, LIFT). Reproducibility was satisfactory for each assay. Marked differences were observed with regard to sensitivities. The LIFT appeared to be the most sensitive assay for detecting anti-HLA alloantibodies and the SPRCA the least sensitive method. For the demonstration of non-HLA platelet-reactive antibodies, test results indicate superior sensitivity for the PIFT as compared to ELISA and SPRCA. Frozen-thawed test platelets could be stored for at least 6 months without loss of reactivity in the ELISA.

We conclude that the flow cytometric combined platelet and lymphocyte immunofluorescence assay is the superior assay for the detection of platelet-reactive antibodies as it is the most sensitive test and at the same time gives additional information on the specificities of the antibodies involved. The ELISA, being a relatively rapid assay, can be considered as a useful alternative, especially when there is a need to use stored platelets.

INTRODUCTION

A variety of assays, frequently as sandwich methods, have been developed for the detection of platelet-reactive antibodies. To determine the amount of platelet-bound immunoglobulins, the test platelets are incubated with an anti-immunoglobulin antibody conjugated with an enzyme, fluorescent or radioactive label. Based on this principle, in our laboratory a solid-phase enzyme-linked immunoassay[1] and a platelet (and granulocyte and lymfocyte) immunofluorescence test with flow cytometric analysis[2] have been introduced for the detection of platelet-reactive antibodies. Recently, a test for the detection of platelet-bound immunoglobulins using solid-phase coated platelets and indicator red cells labeled with anti-IgG, has become commercially available[3]. The relative values of these different assays although of pratical importance are not known. We have compared the qualitative performances of these three methods.

MATERIALS AND METHODS

Solid phase red cell adherence test (SPRCA)

The solid phase red cell adherence test is a commercially available test kit (Modified Capture P, Immucor, Norcross, GA, USA) and was performed according to the manufacturer's instruction. Platelet suspensions were prepared by differential centrifugation of EDTA-anticoagulated blood samples, washed five times with the Modified Capture P (MCP)-Platelet Wash and Storage Solution (PWSS) and resuspended in PWSS at a concentration of 50 x 10^9/L. A platelet monolayer was prepared by adding 5 x 10^6 platelets to each well of the microplate (MCP). The plate was centrifuged (5 min. at 200 g) and washed with 0.9% NaCl six times. To each well 2 drops of low-ionic strength solution (MCP) were added, followed by 50 microliters of serum. After incubation (30 min at 37°C), the plates were washed six times and 1 drop of indicator Red Cells (MCP) was added to each well, followed by centrifugation (1 min. at 800 g.) without breaking. Reactions were read visually, compared with the reactions obtained with the control sera included in the kit and scored on a 5 point scale: 1 =

negative; 2 = doubtful negative; 3 = weakly positive; 4 = positive; 5 = strongly positive. Tests were read by 3 independent observers (in 523 cases) or 2 independent observers (in 59 cases). Mean scores were used as test outcome. Mean scores equal to or greater than 3 were considered as positive.

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay was performed as previously described with minor modifications[1]. Platelets were isolated by differential centrifugation from EDTA-anticoagulated blood samples, washed 4 times in a solution of 0.009 M Na_2EDTA in 0.013 M phosphate-buffered 0.145 M NaCl, pH 7.4 (EDTA-PBS) and resuspended in EDTA-PBS (100 x 10^9/l). A platelet monolayer was prepared by adding 5 x 10^6 platelets to the wells of a microtitre strip. The strips were centrifuged (5 min at 300 *g*), incubated for 15 min at 37°C and washed with PBS-Tween (0.05% v/v) four times. To each well 50 μl of serum were added and incubated for 60 min at 37°C. All test and control sera were prediluted 1:10 with PBS-Tween-BSA (4%) to reduce aspecific binding of immunoglobulins. After four wash cycles, 100 μl of protein-G conjugated to horseradish peroxidase (Zymed, Zymed Laboratories Inc., San Francisco, USA) diluted 1:10,000 in PBS-Tween-BSA were added and incubated for 30 min at 37°C. After washing four times, 100 μl of a freshly prepared substrate solution (80 mg of orthophenylene-diamine in 50 ml citrate/phosphate buffer 0.025 M/0.04 M, pH 5.0, to which 20μl 30% H_2O_2 is added just before use) were added. The plates were placed at room temperature (RT) in the dark and after 10 min. the enzyme reaction was stopped by adding 50 μl 4 N H_2SO_4. Optical densities (O.D.) of the wells were read with an automated micro-ELISA-reader (Titertek, Multiscan MC) at 492 nm (against 620 nm. dual wave length). All tests were performed in triplicate and mean values were used for further calculations. The test result was expressed as the ratio of the O.D. value of a test serum to the O.D. value of a standard negative control serum. Based on results from experiments with negative control sera, tests were considered positive when the ratio was 1.2 or more.

Platelet and lymphocyte immunofluorescence tests (PIFT/LIFT)

The flow cytometric immunofluorescence test was performed as described with some modifications[2]. Platelets, granulocytes and lymphocytes were isolated from EDTA-anticoagulated blood samples by differential centrifugation and Ficoll-Hypaque density gradient separation. Platelets were fixed with paraformaldehyde (1%). After washing and resuspension in EDTA-PBS-BSA, the cell concentrations were adjusted to 50 x 10^9/l for platelets and 1.5 x 10^9/l for mononuclear cells and granulocytes in the final mixed cell suspension. Fifty μl of the mixed cell suspension were incubated with 50 μl of serum (30 min. at RT). The cells were then washed three times and 50 μl of a 1:40 dilution of goat $F(ab')_2$-anti-human IgG conjugated with fluorescein-isothiocyanate (FITC) (Dakopatts, Glostrup, Denmark) were added for 30 min. at RT. Finally, the cells were washed and fluorescence was measured using a flow cytometer (FACScan, Becton and Dickinson Immunocytometry Systems (BDIS), San Jose, CA). Standard gates were set around the platelets, granulocytes and lymphocytes on the basis of their forward and sideways light scatter characteristics and histogram analyses of FITC-fluorescence were performed using fixed markers to distinguish negative and positive events. All tests were performed in duplicate. To improve the quality of the analysis, 10 μl of a mixture of LDS-751 and propidium-iodide (2 μg/ml resp. 0.1 μg/ml in PBS) was added 10-60 min. before analysis[4]. All tests were performed in duplicate and mean values were used as test results. A test result was scored 'positive' when the percentages of positive cells were greater than 25% and 35 % for PIFT and LIFT, respectively. These values were choosen based on the results obtained from experiments with negative control sera. A test result was rejected when the difference between the duplicate values exceeded 15% and both values did not give the same test result in terms of being positive or negative (PIFT: 6/354 cases; LIFT 6/354 cases).

Storage of platelets

For use in the SPRCA, the platelets were stored in PWSS at a concentration of 50 x 10^9/L at 4°C until use. For use in the ELISA, 120 μl of ice cold EDTA-PBS-DMSO 10% were added to each well of freshly prepared platelet monolayers and the cells were frozen by immediately placing the strips in a

freezer at -70°C. Before use the plates were thawed in a water bath at 37°C, and immediately washed four times.

Test sera and test cells

Patient sera were used with the following reactivities: two sera with broadly-reactive (> 90% of random donor cells) anti-HLA antibodies obtained from two multitransfused patients; one serum with anti-HPA-1a antibodies from a patient with neonatal alloimmune thrombocytopenia; one serum with anti-HPA-5b antibodies from a multitransfused patient; two sera from patients with the clinical diagnosis of autoimmune thrombocytopenia (AITP); one serum with EDTA-dependent antibodies; three sera containing IgG-anti-A, one serum obtained from a patient with a hematological malignancy and two sera from healthy donors (IgG-anti-A titers 320; 16 and 128). Negative control sera were obtained from untransfused male blood donors. All sera were stored at -70°C until use. A panel of 6 donor cells selected for HPA-1a and HPA-5b antigen composition and ABO-type was used.

Statistical analysis

The reproducibility of the various assays was determined by comparison of 118 test results obtained on 3 different occasions. The reproducibility of the ELISA is expressed as the mean coefficient of variation (CV), determined as follows. For each set of triplicate values the standard deviation (SD) of the natural logarithm of the ELISA result was calculated. These SD's showed no correlation with the means of the triplicate values. The CV was then calculated as the antilog of the mean of these SD's. Test results of PIFT and LIFT were expressed as percentage positive cells and measured on a scale between 0 and 100. For these measures a CV is not appropriate. The SD's of the triplicate values of the PIFT and LIFT were, as expected, smaller for PIFT and LIFT outcomes near 0 and 100 percent. Therefore, these SD's were calculated after application of an arcsin transformation. The reported mean SD for each triplicate value for both PIFT and LIFT corresponded with the SD for values of PIFT or LIFT near 50%. The SD for values x closer to 0% and 100% may be found by multiplication with the factor $2(x(1-x))^{0.5}$. SPRCA test results were expressed as negative or positive. The reproducibility of this assay was analyzed by determining the concordance of

the repeat test results. This method was also applied to ELISA, PIFT and LIFT after dichotomization of each test result as positive or negative.

RESULTS

Reproducibility of SPRCA, ELISA, PIFT and LIFT

In three separate experiments the test sera and 3 negative control sera were tested against freshly prepared platelets from the same 2 - 6 donors. This resulted in 58 cell-serum pairs in which undiluted serum was used. Three of the sera were also tested in 10 serial doubling dilutions against cells from 2 of the donors. Thus, a total number of 118 cell-serum pairs were tested in each experiment and evaluated for reproducibility. The mean CV of the ELISA results from the 3 experiments was 7.9%. The average SD of the PIFT and LIFT between these 3 experiments was 6.2% and 3.6% respectively. Table I shows the results classified as concordant or discordant. The percentages of concordant results within each of the individual assays were: PIFT 92%, LIFT 93%, SPRCA 86% and ELISA 85%. The reproducibility of the assays in terms of being positive or negative depended on the level of reactivity of the cell-serum pairs. For the SPRCA, no discrepant results were seen for tests with strongly positive reactions (score 5) while discrepancies were observed between negative and weakly positive tests in 15/109 (14%) of cases following repeat SPRCA. For the ELISA, tests with a ratio above 1.6 were always positive on repeat testing (n=13), the discrepancies occurred for test results with values between 0.7 and 1.6 in 18/105 of cases (17%). Also, in the immunofluorescence assays, tests with a relatively high percentage of positive cells (PIFT >50%; LIFT >40%) were positive on repeat occasions, whereas tests with a low percentage of positive cells (PIFT <14%; LIFT <25%) were and remained consistently negative. Discrepant results were observed in the PIFT for values between 14% and 50% in 9/52 cases (17%), and in the LIFT for values between 25% and 40% in 9/48 cases (19%).

Table I. Reproducibility of SPRCA, ELISA, PIFT and LIFT

Experiment			SPRCA	ELISA	PIFT	LIFT
1	2	3				
+	+	+	12	21	24	25
—	—	—	89	79	79	78
+	—	—	12	10	5	5
+	+	—	3	8	4	4
n.e.*			2	0	6	6

In 3 separate experiments freshly prepared donor platelets were tested against the same set of selected anti-sera (see materials and methods). Test results were scored as +: positive or —: negative.
Figures indicate the number of observations.
* n.e.: not evaluable.

Detection of different types of antibodies by SPRCA, ELISA, PIFT and LIFT

To establish the frequency of falsely positive test results 3 negative control sera were tested against 6 donor cells on 3 separate occasions. No falsely positive tests were observed for SPRCA and ELISA, whereas only one falsely positive reaction was seen with the PIFT and LIFT. To evaluate the ability of the various assays to demonstrate the presence of different types of antibodies, the selected sera with various antibodies were tested against fresh test platelets from the selected donor panel.

Anti-HLA antibodies. Two sera containing anti-HLA antibodies were tested against cells from 2 donors on 3 occasions. ELISA, PIFT and LIFT gave consistently positive test results, whereas the SPRCA yielded one falsely negative result.

Anti-HPA antibodies. A serum containing anti-HPA-1a (Zw^a) antibodies tested on 3 occasions against cells from 4 HPA-1a positive donors, gave always positive results in PIFT, ELISA and SPRCA. To assay

for false positivity, the same serum specimen was also tested against 2 cells from HPA-1a negative donors. The results were in all instances negative. A serum with anti-HPA-5b (Br^a) antibodies tested against 3 HPA-5b positive cells at 3 occasions, showed positive test results obtained on 7 of 8 occasions (1 test not evaluable) in the PIFT, on 8 of 9 occasions in the ELISA and only on 2 of 9 occasions in the SPRCA. This serum was at the same time tested against 3 HPA-5a negative cells. ELISA and SPRCA results were always negative, one falsely positive result was noted in the PIFT.

Platelet-reactive auto-antibodies. Two sera from patients with AITP were tested against cells of 2 donors on 3 occasions. One serum gave consistently positive reactions in the PIFT against cells from both donors but was negative in the other assays. In the second serum autoantibodies were not clearly demonstrable as the PIFT showed only weakly positive reactions on 2 out of 6 occasions; also the ELISA reacted weakly on 2 out of 6 occasions; the SPRCA was negative. Apparently, platelet auto-antibodies were detected in 1 serum by PIFT only.

EDTA-dependent platelet reactive auto-antibodies. A serum containing EDTA-dependent platelet auto-antibodies was tested against 2 donors in 3 occasions. Only the PIFT was positive in 5/6 tests. The other assays yielded negative results. This indicates greater sensitivity of the PIFT for these antibodies. To exclude that the higher EDTA concentration used in the PIFT (PIFT: 0.027M; ELISA: 0.009; SPRCA: no EDTA) might have caused this result, the PIFT was repeated using 0.009 EDTA with similar results (data not shown).

ABO incompatibility. Three sera containing IgG anti-A antibodies were tested against cells from 2 group A donors on 3 occasions. Anti-A antibodies were detected only in the PIFT, where 2 out of the 3 sera reacted consistently against the cells of 1 donor. These antibodies were not detected by ELISA or SPRCA.

Table II. Comparison of sensitivity of SPRCA, ELISA, PIFT and LIFT

Serum tested	Cell donor	SPRCA	ELISA	PIFT	LIFT
anti-HLA(1)	1	2	8	8	32
	2	2	16	16	64
anti-HLA(2)	1	16	64	64	256
	2	16	8	32	256
anti-HPA-1a	1	2	4	2	—*
	2	2	2	2	—

Three antisera were tested in serial doubling dilutions against freshly prepared platelets form 2 HPA-1a positive donors on 3 occasions.
Values indicate mean titers.
* anti-HPA-1a antibodies are directed against a platelet-specific antigen and thus only detectable in assays using platelets as target cells.

Comparison of sensitivity of SPRCA, ELISA, PIFT and LIFT

To compare the sensitivity of the assays, 2 sera containing anti-HLA-antibodies and 1 serum containing anti-HPA-1a were tested in serial doubling dilutions against anti-HPA-1a positive donor cells on 3 different occasions. Undiluted negative control sera were used for dilution in SPRCA, PIFT and LIFT. For use in the ELISA prediluted control serum (1:10 with EDTA-PBS-BSA) was used. An example of this experiment is shown in figure 1. A summary of all test results showing the mean titers obtained from these 3 experiments is given in Table II. When the relative sensitivities of the assays for the detection of HLA-antibodies are compared it appears that the LIFT is the most sensitive assay to detect HLA antibodies followed by PIFT, ELISA. The SPRCA was the least sensitive test.

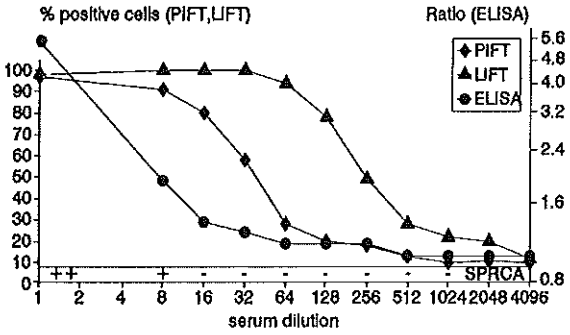

Figure 1. Comparison of sensitivity of PIFT, LIFT, ELISA and SPRCA. Serial dilutions of a serum containing anti-HLA antibodies were tested against platelets of the same donor with each assay.
Ratio (ELISA) = O.D. test serum/O.D. negative control serum (see Materials and Methods).

Effects of storage of test platelets on SPRCA and ELISA

To evaluate the effect of storage of the platelets on the results, platelets were stored for periods of 2 and 6 months and tested against the set of sera as used in the reproducibility experiments. Results were compared with those obtained with freshly prepared cells. For use in SPRCA, platelets were not frozen for storage but preserved in liquid medium (PWSS) in accordance to the manufacturer's directions. For ELISA, frozen/thawed platelets were used. Table III shows that storage of test platelets for 2 months did not significantly alter the level of reactivity of the platelets in SPRCA nor ELISA. However, after extending the storage period to 6 months, all positive reactions in the SPRCA had disappeared, whereas the reactivity as assayed in the ELISA was constant. Falsely positive test results after storage were not observed using the ELISA. Using the SPRCA one of 18 negative control tests became positive after 6 months of storage. Thus, platelets can be stored for at least 6 months for use in the ELISA, whereas platelets stored for use in the SPRCA lose their reactivity between 2 and 6 months of storage.

Table III. Effects of storage of test cells on SPRCA and ELISA

Storage period (mo)		SPRCA			ELISA		
		0	2	6	0	2	6
serum	cell donor						
anti-HLA(1)	1	2	2	—	16	32	16
	2	2	4	—	16	16	16
anti-HLA(2)	1	8	1	—	64	16	16
	2	16	32	—	8	8	32
anti-HPA-1a	1	2	2	—	4	2	4
	2	2	2	—	2	16	4

Three antisera were tested in serial doubling dilutions with freshly prepared platelets and platelets stored for 2 and 6 months from the same 2 HPA-1a positive donors.
Figures indicate titers.

DISCUSSION

In this study we compared four different assays for the detection of platelet-reactive antibodies. Platelet-alloantibodies are involved in a variety of clinical conditions such as immunological platelet refractoriness, neonatal alloimmune trombocytopenia and post-transfusion purpura. Laboratory assays for platelet alloimmunization should be particularly sensitive for HLA-antibodies since HLA-antibodies are the most frequent cause of immunological platelet refractoriness. We demonstrate here that the flow cytometric immunofluorescence tests were the most sensitive assays to detect HLA-antibodies. The LIFT was considerably more sensitive than the PIFT. The ELISA was slightly less sensitive than the PIFT and the SPRCA was significantly less sensitive. The lymphocytotoxic antibody test (LCT) that is frequently used as the standard assay for the detection of HLA-antibodies,

was not included in this study. It has been reported previously that the LIFT is approximately 7 doubling dilution steps more sensitive than the LCT[5]. Considering our observation in this study that the LIFT is 4-5 doubling dilution steps more sensitive than the SPRCA, we assume that the sensitivity of the LCT for HLA-antibodies is well below that of PIFT, LIFT and ELISA and will be comparable to that of the SPRCA or less. This is supported by another observation indicating that the SPRCA is 2 dilution steps more sensitive for HLA antibodies than the LCT[6]. Thus, to monitor for the presence of HLA-antibodies, the flow cytometric combined assays of PIFT and LIFT are first choice. A further advantage of the combination of PIFT and LIFT is that additional information is obtained with regard to the specificity of the antibodies e.g. in the case of HLA-antibodies both PIFT and LIFT will be positive (in the case of weakly reactive antibodies only the LIFT may be positive), whereas in the case of HPA-antibodies only the PIFT will be positive.

In the investigation reported here PIFT, ELISA and SPRCA were equally sensitive to detect anti-HPA-2a-antibodies. Anti-HPA-5b antibodies were frequently missed. Nevertheless, according to the intercomparison the PIFT gave the best results. This was not unexpected as HPA-5b-antibodies are difficult to detect using sandwich-assays because of the low cell surface expression of the antigen involved[7]. In addition to the investigations for HLA- and HPA-antibodies, selected tests were applied to a set of sera containing platelet auto-antibodies and IgG-anti-A antibodies. The results suggest that the PIFT has a superior sensitivity. Also, the PIFT only was able to demonstrate the presence of EDTA-antibodies.

As far as the reproducibility of the individual assays is concerned, the quality of different assays was comparable and satisfactory. Discrepancies between repeated tests were only observed as values close to the threshold values, confirming earlier observations for the ELISA and the PIFT[1]. Obviously, the percentage of discordant results strongly depends on the sample of cell-serum combinations and the definition of the cut-off points for positivity of the assays. For instance, a high prevalence of serum-cell combinations with a test-result around the cut-off point of the assay will lead to a relatively large proportion of discordant results. In comparison to SPRCA and ELISA, the PIFT and LIFT showed the lowest number of discordant results and at the

same time the highest number of positive tests on the same set of sera and cells tested, indicating better reproducibility. An advantage of the use of stored cells is that screening sera for antibodies will consume less time as fresh test cells do not need to be prepared for each test. A second advantage is that selected cells e.g. cells typed for HLA- and/or HPA-antigens or cells from platelet donors for rapid platelet crossmatching can be kept in stock. Earlier, we have reported the usefullness of this approach[8]. Therefore, we studied the possibilities to store platelets for ELISA and SPRCA. For use in SPRCA platelets were stored in the liquid state in the commercially supplied storage medium according to the manufacturers directions. The reactivity of the stored cells was comparable to that of fresh cells after 2 months of storage, but - in contrast to the manufacturers specifications - after a 6 months storage period all reactivity was lost. In contrast, platelets stored frozen retained their reactivity in the ELISA with anti-HLA-antibodies and anti-HPA-1a-antibodies for at least 6 months. A storage procedure for platelets for use in the flow cytometric immunofluorescence procedure has not yet been developed in our laboratory as initial experiments indicated that storage resulted in changes in the scatter parameters interfering with the standardized analysis procedure.

In conclusion, the flow cytometric combined platelet and lymphocyte immunofluorescence assay is the superior assay for the detection of platelet-reactive antibodies as it is the most sensitive test and at the same time gives additional information on the specificities of the antibodies involved. The ELISA, being a relatively rapid assay, can be considered as a useful alternative especially when there is a need to use stored platelets.

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CHAPTER 8

A FLOW CYTOMETRIC PLATELET IMMUNOFLUORESCENCE CROSSMATCH FOR PREDICTING SUCCESSFUL HLA-MATCHED PLATELET TRANSFUSIONS

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SUMMARY

Platelet crossmatching may provide a useful way of selecting donors for effective platelet transfusions in patients refractory to random donor platelet concentrates due to alloimmunization. We assessed the predictive value of a flow cytometric platelet immunofluorescence crossmatch test for the outcome of HLA-matched platelet transfusions in a group of alloimmunized patients. PIFT-crossmatches were performed for 104 HLA-matched platelet transfusions administered to 30 patients. A negative PIFT-crossmatch correctly predicted a successful platelet transfusion (1-hr post-transfusion platelet recovery > 20%) in 56/75 (75%) cases. We also considered non-immunological factors that, in combination with alloimmunization, might have contributed to an unsuccessful transfusion result i.e., fever, septicaemia, splenomegaly, disseminated intravascular coagulation and bleeding. The predictive value of a negative PIFT-crossmatch was better when these non-immunological factors were absent [48/59 (81%) correct predictions] than when these factors were present [8/16 (50%) correct predictions] ($p=0.01$; chi-square test).

The effect of ABO-incompatibility between donor and recipient on the predictive value of the PIFT-crossmatch was also analyzed. Positive PIFT-crossmatches occurred more frequently in ABO-incompatible donor-recipient combinations [in 18/28 (64%) cases] than in ABO-compatible donor-recipient combinations [in 11/76 cases (14%)] ($p<0.001$, chi square test). Successful platelet transfusions were observed on 53/76 (70%) occasions in ABO compatible transfusions as compared to 16/28 (57%) in ABO-incompatible transfusions. This difference was statistically not significant ($p=0.23$; chi square test). Consequently, a negative PIFT-crossmatch appeared to be non predictive for the transfusion outcome in cases of ABO-incompatibility between donor and recipient.

We conclude that the PIFT-crossmatch for platelet donor selection in addition to matching for HLA-antigens, is predictive for the outcome of ABO-compatible transfusions in alloimmunized recipients and gains power of prediction when non-immunological causes for platelet refractoriness are absent.

INTRODUCTION

Following repeated platelet transfusions alloimmunization to random donor platelets may develop. In such conditions efforts are made to provide these alloimmunized patients with platelet concentrates from HLA-matched donors. Unfortunately, a proportion of HLA-matched platelet transfusions are not successful. Previous reports have indicated that the addition of a platelet crossmatch to donor selection according to HLA-matching is of value for predicting the transfusion results[1-8]. In these studies, the question of a possible influence of ABO-incompatibility on the performance of the platelet crossmatch test was not addressed[1,2,3,5,7,8]. In one study 2 patients received major ABO incompatible platelet transfusion with negative crossmatch tests and adequate post-transfusion platelet increments[4]. In a second study it has been reported that ABO-incompatibility between donor and recipient resulted in an increased occurrence of positive ELISA-platelet crossmatches and a decreased frequency of successful platelet transfusions[6].

Furthermore, little is known about the effects of the presence of non-immunological adverse factors that may compromise post-transfusion platelet survival (e.g., fever, septicaemia, splenomegaly, disseminated intravascular coagulation and bleeding) on the predictive value of platelet crossmatch assays. In most studies, platelet transfusions that were given in the presence of these non-immunological adverse factors were excluded from the analysis[1,3,4,5,8]. In other studies such transfusions have been included but no analysis directed at the possible effects of these factors on the value of the platelet crossmatch was presented[2,6,7].

In the present study we have evaluated the performance of a platelet immunofluorescence (PIFT) crossmatch that we have recently developed[9]. This assay differs from the platelet immunofluorescence techniques used in previous studies[1,2,3] in that the test results are measured by flow cytometry in stead of microscopic reading which results in increased sensitivity of the PIFT[9]. We analyzed the outcomes of HLA-matched platelet transfusions in alloimmunized patients and compared these with the results of this flow cytometric platelet immunofluorescence crossmatch and considered the influences of ABO-incompatibility between donor and recipient as well as unfavourable non-immunological factors.

MATERIALS AND METHODS

Patient entry criteria and procedure

Alloimmunized patients were given HLA-matched platelet transfusions. The following patients were entered into the study:

1) patients in whom two or more successive transfusions of random donor platelets resulted in a 1-hr post-transfusion platelet recovery of less than 20% and in whom at that time no clinical factors were present that might account for decreased platelet recoveries (temperature >38.5 °C; septicaemia; splenomegaly; disseminated intravascular coagulation and major haemorrhage) (group I: 22 patients). In 20 out of these 22 patients, HLA-antibodies were detectable in the serum; in one patient platelet specific alloantibodies were detected directed against Human Platelet Antigen (HPA)-5b by the monoclonal antibody immobilized platelet antigen (MAIPA)-assay and in the remaining patient no platelet-reactive antibodies could be demonstrated.
2) patients in whom when a single transfusion of random donor platelets showed a 1-hr post-transfusion platelet recovery of less than 20% and in whom at that time HLA-antibodies had been demonstrated in the patient's serum (group II: 4 patients)
3) patients with broadly-reactive lymphocytotoxic antibodies i.e. reacting with the cells from all donors tested and therefore assumed to be refractory to random donor platelets (group III: 4 patients).

Data were collected on 104 transfusions in 30 patients meeting the patient entry criteria. When HLA-matched platelets were transfused into a patient, a platelet immunofluorescence crossmatch test (PIFT) was performed using platelets from the donor and serum of the patient. The result of the PIFT-crossmatch was then compared with the result of the transfusion in order to see whether the PIFT-crossmatch would predict the transfusion result. Patient sera for crossmatching were generally obtained just before transfusion (in 87 of 104 cases), within 2 days prior to transfusion (3 cases) or within 3 days following the platelet transfusion (14 cases).

Donor selection, HLA-matching and ABO-matching

Donors were selected from an HLA-typed donor pool (Red Cross Blood Bank, Leiden) or were HLA-typed family members (dr. Daniel den Hoed Cancer Center). The degree of HLA-matching with regard to the HLA Class I antigens (A- and B-locus) was scored as described by Duquesnoy et al.[10] as follows: A match: all 4 antigens in donor identical to those of recipient; B1U: only 3 antigens detected in donor; all present in recipient; B2U: only 2 antigens detected in donor; all present in recipient; B1X: three donor antigens identical to recipient; fourth antigen cross-reactive with recipient; B2X: two donor antigens identical to recipient; third and fourth antigen cross-reactive with recipient; C: one antigen of donor not present in recipient and non cross-reactive with recipient. The frequencies of the various match categories were: A: n=7; B1U: n=13; B2U:n=60; B1X: n=12; B2X: n=1; C: n=11. Thus, in 80 transfusions no HLA-incompatibilities were present between donor and recipient, in 13 transfusions incompatibility for cross-reactive HLA-antigens was involved and in 11 transfusions 1 major HLA-mismatch was present. The donor platelets were ABO-compatible with the recipient in 76 transfusions and ABO-incompatible in 28 cases.

Platelet Products

Platelets were collected by plateletapheresis using a Haemonetics V50 cell-separator (Red Cross Blood Bank Leiden, 91 procedures; Dr Daniel den Hoed Cancer Center, 13 procedures). Platelets were collected in a closed system, allowing storage of platelets for up to 5 days. Eighty-nine transfusions were administered within 1 day following plateletapheresis, 13 products had been stored for 2 to 3 days before transfusion and 2 products were transfused at 4 - 5 days following collection. The age of the platelet product did not correlate with transfusion outcome. The mean 1-hr post-transfusion platelet recovery was 36% $\pm$ 25 (sd) for platelet products transfused less than 2 days old and 37% $\pm$ 39 for platelet products that were 2-5 days old at the time of transfusion. Platelet products were filtered to remove the contaminating leukocytes when HLA-antigens were present on the donor cells that were either cross-reactive with recipient HLA-antigens or absent in the recipient. In the case of ABO-incompatibility between donor plasma and the recipient's red cells, the products were concentrated to a final

volume of 40-50 ml to reduce the amount of ABO-incompatible plasma. For this purpose acid-citrate-dextrose, formula A was added to the platelet product to lower the pH to approximately 6.5 and a second centrifugation step was performed. As a result the platelet pellet could immediately be resuspended after centrifugation. The volume reduced platelet concentrates were transfused within one hour following preparation. The numbers of transfused platelets were always determined. The median number of platelets transfused was 293 x 10^9 (range 154-567).

Documentation of platelet transfusion results

Patient platelet counts were determined prior to transfusion and at 1-hr after transfusion. Post-transfusion platelet recoveries were then calculated using the formula:

$$\text{Recovery (\%)} = 100 \times \frac{\text{platelet increment x blood volume}}{\text{number of transfused platelets}}$$

Platelet increment: platelet count at 1 hr after transfusion minus platelet count prior to transfusion. Blood volume (L) was established as 2.5 x body surface area (m^2)[11]. A transfusion was considered successful when the 1-hr post-transfusion platelet recovery was greater than 20 %. At the time of each transfusion, the presence or absence of the following conditions that might reduce platelet survival were documented: temperature, septicaemia, splenomegaly, disseminated intravascular coagulation and major haemorrhage (a decrease of hemoglobin value of 2 mmol/l or more within 24 hrs)[12-17].

Serological assays used for the detection of HLA- and platelet reactive antibodies

The following serological assays were used to demonstrate HLA- and other platelet reactive antibodies:

a: the combined platelet and lymphocyte immunofluorescence test (PIFT, LIFT) with flow cytometric analysis, according to Sintnicolaas et al.[9]. Based on a large number of tests with negative control sera, the PIFT-crossmatch was scored positive when the percentage of positive cells

was greater than 25%.

b: the monoclonal antibody immobilized platelet antigen assay (MAIPA)[18] using the following monoclonal antibodies: anti-HLA (w6/32, Dakopatts, Glostrup, Denmark), anti-GP Ia (thromb/1, Central Laboratory of the Dutch Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), anti-GP IIIa (thromb/4, CLB, Amsterdam, The Netherlands).

c: the standard lymphocytotoxicity test (LCT) with eosine dye exclusion[19].

To examine patient sera for the presence of HLA- and platelet-reactive allo-antibodies, the LCT, PIFT and LIFT were used and the serum was tested against a panel of cells from 10 (LCT) or 5 (PIFT and LIFT) randomly chosen donors. HLA-antibodies were considered to be present when at least 2 positive reactions were observed in LCT and/or PIFT and LIFT.

For platelet crossmatching the PIFT was used. When ABO-incompatibility was present between the donor cells and the patient serum, the platelet crossmatch was also performed with patient serum from which the relevant anti-A,-B antibodies had been absorped by incubation with beads coated with A or B blood group antigens (Synsorb A/B, Chembiomed, Edmonton, Alberta, Canada). Also, in the majority of cases a MAIPA-crossmatch was performed using the monoclonal antibodies as described above.

Statistical Methods

To test for differences in the frequencies of successful transfusions in relation to platelet crossmatch results, ABO-matching and the presence or absence of clinical factors associated with decreased post-transfusion platelet recoveries, the Pearson chi-square test was used.

RESULTS

Patients

Thirty patients with severe thrombocytopenia due to bone marrow depression or insufficiency who required platelet transfusions, were studied. Patient characteristics are shown in Table I.

Predictive value of the PIFT

The ability of the PIFT to predict the transfusion outcome of HLA-matched platelets is shown in Table II. When the PIFT-crossmatch was negative, platelet transfusions were successful on 56 of 75 (75%) occasions. In comparison, transfusions with a positive PIFT crossmatch were successful in

Table I. Patient Characteristics

	Group*			
	I	II	III	Total
age (median,(range))	55 (23-80)	63 (47-65)	50 (43-55)	54 (23-80)
sex (male/female)	10/12	2/2	1/3	13/17
diagnosis				
ANLL	11	-	1	12
ALL	1	-	-	1
CML	1	-	2	3
MDS	3	1	-	4
NHL	-	1	1	2
Waldenström's disease	-	1	-	1
Multiple myeloma	1	-	-	1
PNH	-	1	-	1
Fanconi's anemia	1	-	-	1
Aplastic anemia	2	-	-	2
Solid tumor	2	-	-	2

ANLL: acute non-lymphocytic leukemia; ALL: acute lymphocytic leukemia; CML: chronic myelocytic leukemia; MDS: myelodysplastic syndrome; NHL: non Hodgkin's lymphoma; PNH: paroxysmal nocturnal hemoglobinuria.
* Group: see Materials and Methods.

13 of 29 (45%) cases. Thus, platelet transfusions with a negative PIFT-crossmatch were more frequently successful than those with a positive PIFT-crossmatch ($p=0.004$, chi square test).

Role of ABO-incompatibility

To determine as to whether ABO-compatibility between platelet donor and transfusion recipient had an effect on the transfusion outcome and influenced the predictive value of the PIFT, the results obtained from ABO-compatible (i.e. cell compatible, plasma compatible or incompatible) ($n=76$) transfusions were compared with the results from ABO incompatible (i.e. cell incompatible) ($n=28$) transfusions (Table II). Fifty-three of 76 (70%) ABO-compatible transfusions were successful as compared to 16 of 28 (57%) ABO-incompatible transfusions ($p=0.23$). Thus, ABO-incompatible platelet transfusions were not significantly less successful than ABO-compatible platelet transfusions. The incidence of positive PIFT-crossmatches was 11/76 (14%) in ABO compatible transfusions versus 18/28 (64%) in ABO-incompatible transfusions ($p<0.001$). Thus, ABO incompatibility between donor and recipient was associated with an increased frequency of positive PIFT-crossmatches. Among ABO-compatible transfusions a negative PIFT-

Table II. Predictive value of PIFT-crossmatching for HLA-matched platelet transfusions: role of ABO-matching

ABO-match	PIFT-Xm	no. of successful Tx/ total no. of Tx (%)		p-value*
all cases	neg	56/75	(75)	0.004
	pos	13/29	(45)	
compatible	neg	49/65	(75)	0.009
	pos	4/11	(36)	
incompatible	neg	7/10	(70)	0.31
	pos	9/18	(50)	

Abbrevations: PIFT-Xm=PIFT-crossmatch; neg=negative; pos=positive; Tx=platelet transfusion.

* p-values calculated by chi-square test.

Table III. Adsorption of relevant anti-A and anti-B antibodies from serum used for platelet crossmatching. Effect on the predictive value of the PIFT-crossmatch for ABO-incompatible HLA-matched platelet transfusions

Adsorption of anti-A, -B antibodies	no. of successful Tx/ total no. of Tx (%)		p-value
	PIFT-Xm negative	PIFT-Xm positive	
no	7/10 (70)	9/18 (50)	0.31
yes	9/14 (64)	6/10 (60)	0.24

Abbrevations: Tx = platelet transfusion; PIFT-Xm = PIFT-crossmatch.

crossmatch was in 49 of 65 cases (75%) associated with successful transfusion results, whereas transfusions with a positive PIFT-crossmatch were successful in 4 of 11 (36%) transfusions only ($p=0.009$). In contrast, among ABO-incompatible transfusions the PIFT-crossmatch had lost its predictability for the transfusion result. Of 10 cases with a negative PIFT-crossmatch, 7 transfusions were successful as compared to 9 successful transfusions of 18 cases with a positive PIFT-crossmatch ($p=0.31$). In 24 of the 28 ABO-incompatible platelet transfusions, the PIFT-crossmatch was also performed with patient sera after absorption of the relevant anti-A,-B antibodies. Of 15 PIFT-crossmatches that had been positive before adsorption, five became negative after adsorption. Nine initially negative crossmatches remained negative. The effect of this adsorption procedure on the predictive value of the PIFT-crossmatch is shown in Table III. It appeared that adsorption of the relevant anti-A,-B antibody did not improve the predictive value of a negative PIFT-crossmatch in ABO-incompatible HLA-matched platelet transfusions.

Table IV. Predictive value of PIFT-crossmatching for HLA-matched platelet transfusions: influence of clinical factors associated with decreased post-transfusion platelet recoveries

Clinical factors*	PIFT- Xm	no. of successful Tx/ total no. of Tx (%)		p-value
all cases	neg	56/75	(75)	0.004
	pos	13/29	(45)	
absent	neg	48/59	(81)	0.02
	pos	13/23	(57)	
present	neg	8/16	(50)	0.03
	pos	0/6	(0)	

Abbrevations: PIFT-Xm=PIFT-crossmatch; neg=negative; pos=positive; Tx=platelet transfusion.
*Clinical factors: temperature>38.5 °C, septicemia, splenomegaly, disseminated intravascular coagulation, major hemorrhage.

Effects of non-immunological causes for platelet refractoriness

Several clinical conditions reported to be associated with decreased post-transfusion platelet survival, were evaluated (see Materials and Methods) and were involved in 22 of 104 (21%) transfusions. A single adverse clinical factor was present in case of 20 transfusions as follows: temperature >38.5°C: n=10; splenomegaly: n=7; disseminated intravascular coagulation: n=2; septicaemia: n=1. At the time of 2 transfusions two factors were concomittantly present i.e., splenomegaly and disseminated intravascular coagulation in the case of one transfusion, and septicaemia plus temperature >38.5°C in the case of a second transfusion. When these adverse clinical factors were present, platelet transfusions were successful in 8 of 22 cases (36%), which compared to a transfusion success in 61 of 82 (74%) cases in the absence of these conditions (p=0.001). We analyzed the data to investigate as to whether the predictive value of the PIFT-crossmatch was influenced by the presence of clinical factors (Table IV). In the absence of the unfavourable clinical variables, a negative PIFT-crossmatch predicted a successful transfusion result in 48 out of 59 (81%) occasions, whereas a positive PIFT was associated with transfusion success in 10 out of 23 cases (43%) (p=0.02). In cases where the unfavourable clinical factors were

present, a negative PIFT-crossmatch predicted a successful transfusion outcome on 8 out of 16 (50%) occasions while in none of 6 transfusions with a positive PIFT crossmatch a successful transfusion was observed ($p=0.03$). This difference in the negative predictive values i.e., 48/59 correct predictions in the absence of non-immunological factors versus 10/23 correct predictions in the presence of non-immunological factors, was statistically significant ($p=0.01$). Thus, it appears that the PIFT-crossmatch maintains its predictive value when clinical conditions potentially interfering with the outcome of transfusion, are involved.

Identification of the cause of positive PIFT-crossmatches

In an attempt to identify the alloantibodies causing positive PIFT-crossmatches additional MAIPA-crossmatches with various monoclonal antibodies were performed. The MAIPA-crossmatch for the detection of HLA-antibodies (monoclonal antibody w6/32), was performed in 95 transfusions. A positive test result was obtained on 4 occasions. In 3 of these, HLA mismatches between donor and recipient were present (C-match: $n=2$; B1X-match: $n=1$), the PIFT-crossmatches were also positive and two of the platelet transfusions were failures. The remaining MAIPA crossmatch was very weakly positive, the PIFT-crossmatch was positive due to ABH-antibodies and the platelet transfusion was successful.

The MAIPA-crossmatch for the detection of anti-HPA-5 antibodies (monoclonal antibody thromb/1) was performed in 94 transfusions: in the case of one transfusion the crossmatch was positive, the HLA-match was B2U, the PIFT crossmatch was negative and the transfusion was unsuccessful. Further analysis using a panel of HPA-typed donor cells, confirmed the presence of anti-HPA-5b antibodies in this patient.

The MAIPA-crossmatch for antibodies directed against antigens on platelet glycoproteins IIb/IIIa (monoclonal antibody thromb/4) was performed in 94 transfusions and was found positive in 5 of 6 transfusions given to the same patient. The PIFT-crossmatch, after adsorption of anti-ABH antibodies was negative in 2 transfusions but positive in 3 transfusions. All transfusions were successful, indicating unexplained positivity of the MAIPA-crossmatch.

DISCUSSION

Transfusion of compatible platelets allows for still effective platelet transfusions when alloimmunization to random donor platelets has occurred. Therefore, it is important to select immunologically compatible donor platelets for subsequent platelet transfusions. The supply of HLA-matched platelets to alloimmunized patients is logistically cumbersome as selected donors need to be called in and platelets have to be collected through plateletapheresis. The process of donor selection should have maximal efficiency and the resulting platelet transfusion a maximal probability of success. The selection of platelet donors based on matching for HLA-antigens only, is still associated with transfusion failures in 20-63% of cases[1-8]. Here we have evaluated the predictive value of an immuno-fluorescence platelet crossmatch performed in addition to selection by HLA-matching. Flow cytometry was used to measure the fluorescence intensities of the tests as this is more sensitive and more objective than microscopic evaluation[9]. Our results indicate that platelet transfusions with a negative PIFT-crossmatch had significantly better outcomes than those with a positive crossmatch. The routine application of the PIFT-crossmatch in the process of donor selection would have prevented 16 of 35 (46%) of the platelet transfusion failures.

A major factor in determining the platelet transfusions result was the presence of clinical factors that decrease post-transfusion platelet survival. The predictive value of the PIFT-crossmatch was maintained when these clinical factors were present. However, a large proportion of transfusions in the presence of these clinical factors (16/22; 73%) were not successful, even with a a negative PIFT-crossmatch. The presence of ABO-incompatibility between platelet donor and recipient did not reduce the frequency of successful transfusions. However, in conditions of ABO-incompatibility the frequency of positive PIFT crossmatches was found to be greater. As a result, the PIFT-crossmatch lost its predictive power in case of ABO-incompatible transfusions. For optimal use of platelet crossmatching, one would probably need to recommend to recruit donors for an HLA-typed donor pool from blood group O blood donors, if possible.

It is not always clear which types of antibodies are causing a positive PIFT-

crossmatch. The MAIPA-crossmatches that were performed were not informative in most cases. One possible explanation is that the PIFT is more sensitive than the MAIPA. Another explanation might be that circulating immune complexes are present[20] which may be detected by PIFT but not MAIPA.

The PIFT-crossmatch for routine donor selection has certain practical disadvantages. For instance, cells from the donors are needed and the selection procedure takes considerably more time. Therefore, we routinely perform platelet crossmatches only in those patients in whom platelet transfusions from HLA-matched donors had failed. The application of a flow cytometric PIFT-crossmatch to select among HLA-matched platelet donors appears presently the procedure of choice.

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CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

9.1 Prevention of refractoriness to random donor platelets due to HLA-alloimmunization

When platelet transfusion therapy was introduced, HLA-alloimmunization appeared a major cause of refractoriness to random donor platelets. In early studies, the reported incidence of HLA-alloimmunization varied from 30-100%[1-6]. At that time patients received blood products that were prepared without additional measures to remove the contaminating leukocytes i.e., platelet concentrates contained approximately $2x10^9$ leukocytes per transfusion and red blood cell concentrates (RBC) $1\text{-}3x10^9$ leukocytes per product[7-8]. As it became clear that the presence of leukocytes in these blood products is the major immunogenic factor for the development of HLA-antibodies, attempts were made to produce leukocyte depleted blood products. Filters were developed to remove the leukocytes from RBC products[9]. As these filters also retained platelets, these could not be used for leukocyte depletion of platelet products. Therefore, leukocyte depletion of platelet products was done at first by differential centrifugation[8]. Subsequently, blood filters have been developed that efficiently and reliably removed leukocytes from platelet products[10]. In a number of prospective randomized studies the effects of leukocyte depletion of blood products on the incidence of HLA alloimmunization have been evaluated[11-15]. The available data clearly indicate that the presently available blood filters result in a degree of leukocyte depletion both from platelet products and from red blood cell concentrates, that is sufficient to prevent the induction of primary HLA alloimmunization. From these studies it is not possible to conclude how many leukocytes are needed for primary HLA alloimmunization. Marwijk Kooy et al.[14] specifically addressed the subject of primary HLA alloimmunization and showed that the use of platelet concentrates and RBC containing less than $5x10^6$ leukocytes per blood product reduced the incidence of primary HLA alloimmunization to 7%. In two other studies blood products containing higher numbers of leukocytes have been used. In one study[12] platelet products contained a mean number of leukocytes of $47\text{-}151x10^6$. In a second study[13] the mean number of leukocytes in RBC was $50x10^6$. In both studies a statistically significant decreased incidence of HLA-alloimmunization was observed i.e., 12%[12] and 10% respectively[13]. Thus,

although the precise number and type of leukocytes necessary for primary HLA alloimmunization, cannot be derived from these studies, it may be concluded that reduction of the leukocyte contamination of blood products to less than $5x10^6$ leukocytes per transfusion will almost completely prevent primary HLA-alloimmunization.

In contrast, the results of several prospective non-randomized studies indicate that the secondary booster HLA antibody response is not prevented using leukocyte depleted blood products. Brand et al.[16] tranfused platelet concentrates containing less than $20x10^6$ leukocytes and RBC containing less than 0.5 $x10^6$ leukocytes. HLA-antibodies developed in 19 of 71 (27%) of females with previous pregnancies. In another study[17] the average leukocyte contamination was $9x10^6$ leukocytes per RBC and $46x10^6$ leukocytes per platelet transfusion. Using these products no primary HLA-alloimmuization occurred in 17 patients, whereas secondary HLA-alloimmunization occurred in 3 of 8 (38%) cases.

In a prospective, randomized study, presented in Chapter 2, we have shown that vigorous leukocyte depletion to a mean of $2x10^6$ leukocytes ($<5x10^6$) per platelet product and less than $1x10^6$ leukocytes per RBC did not result in the prevention of secondary HLA-alloimmunization. Using these leukocyte depleted blood products approximately 40% of the patients developed HLA-antibodies and 25% of the patients became refractory to random donor platelets. These frequencies are similar to those observed in the group of patients receiving non-leukocyte depleted platelet products. Thus, while with presently available leukocyte depletion techniques the problem of primary HLA-alloimmunization cna be avoided, the incidence of apparently secondary HLA alloimmunization is not modified. The latter phenomenon remains a major clinical problem. The same conclusion was reached in a recent prospective non-randomized study on the effectiveness of prestorage filtered pooled random donor platelet concentrates. In a large group of patients primary HLA alloimmunization occurred in only 3 of 112 (3%) patients whereas secondary HLA alloimmunization was observed in 16 of 52 (31%) of cases[18]. In order to reduce further the incidence of HLA-alloimmunization future studies would need to focus especially on patients that are at high risk of secondary HLA antibody formation e.g., females with previous pregnancies and patients with previous non leukocyte depleted

blood transfusions and/or transplants.

For future studies on this subject, various approaches may be considered.
UV-B irradiation interferes with the function of the antigen presenting cells (APC's). In particular, the ability of APC's to stimulate allogeneic mononuclear cells is abolished by UV-B treatment. Therefore, UV-B irradiation of platelet products could be useful to prevent alloimmunization. An experimental study in dogs showed that only 1 of 12 (8%) of dogs became immunized following transfusion of UV-B treated platelets versus 18 of 21 (86%) dogs receiving untreated platelets[19]. However, in a recent clinical study in patients undergoing cardiopulmonary bypass, UV-B irradiation of platelet concentrates was found to be ineffective in preventing primary HLA-alloimmunization[20]. On the basis of in vitro studies it has been postulated that UV-B irradiation might be effective in the prevention of secondary alloimunization[21]. Clinical data on this specific subject have not been reported yet.
Another possibile approach is the reduction of the plasma content of platelet concentrates. Transfusion of plasma may influence HLA-alloimmunization in some way perhaps through the presence of soluble HLA-antigens[22-24].
Another approach that might be considered to prevent secondary HLA-alloimmunization is the selection of platelet donors sharing HLA Class II DR-antigens with the recipient. The sharing of a DR-antigen between donor and recipient might lead to a state of individual immunological tolerance as suggested by data in renal and cardiac transplantation patients[25]. No studies on platelet transfusion have been performed so far and such studies would be logistically complex as both HLA-Class II typed RBC and platelet concentrates have to be transfused.
Finally, one may consider to investigate the effects of a more vigorous leukocyte depletion technique resulting in a decrease towards e.g. 10^3-10^4 leukocytes per transfusion. The presently available types of leukocyte filters do not reach this degree of leukocyte reduction. We have evaluated the performance of 4 different leukocyte removal filters for platelet products and found a 2-3 $\log_{10}$ leukocyte reduction (Chapter 3). At present, techniques for 5-6 $\log_{10}$ leukocyte depletion are being developed for routine application[26]. A practical problem for the quality control of blood products containing only

10^3-10^4 leukocytes is that the detection limit for accurate leukocyte counting is approximately 10^6/L for manual counting techniques and $0.1x10^6$/L for flow cytometric methods and that these admixtures are difficult to assess[27]. Because DQ-A genes are present in leukocytes but not in platelets, PCR-techniques amplifying the DQ-A DNA sequences are being developed for the quantification of these low numbers of leukocytes[28]. A second problem is that it might be useful not only to know the number of leukocytes present in blood products but also which cell types are involved as especially the antigen presenting cells are involved in primary HLA-alloimmunization. Again, with leukocyte numbers below 10^4 per blood products, these types of analyses will be hampered by technical limitations.
It remains to be seen whether an approach of more vigorous white cell elimination will be effective in preventing secondary HLA alloimmunization. Some would argue that secondary HLA-alloimmunization may be the result of transfusion of pure platelets alone and that more vigorous depletion will never be sucessful.

9.2 Serological techniques for the demonstration of platelet-reactive alloantibodies

Sensitive and specific assays for platelet alloantibodies are valuable in platelet transfusion therapy as these tests may be used to discriminate between immunological and non-immunological causes for platelet refractoriness. Also, such assays are useful for selecting platelet donors for alloimmunized patients. A brief review of these assays has already been presented in the introductory Chapter. Several assays have been studied and compared and presented in this thesis.
The ^{51}Cr-lysis assay (Chapter 4) was found to detect platelet alloantibodies and to be predictive for platelet transfusion results. A disadvantage of this test is that non-complement activating antibodies are not detected; moreover, radioactive labels and fresh test platelets are required.
Assays measuring platelet-bindable-immunoglobulins may also detect non-complement binding alloantibodies; by using an enzyme label or a fluorescent label the use of radioactivity is avoided.
An ELISA-assay for the detection of platelet alloantibodies has been

developed and described in Chapter 5 and the results appear of similar quality as those obtained with a microscopic platelet immunofluorescence assay. The ELISA is much easier to handle. Further, it can be performed with frozen stored test platelets.

With the development of flow cytometry, it has become possible to apply the latter technique for routine platelet, granulocyte and lymphocyte immunofluorescence tests (PIFT, GIFT, LIFT). In Chapter 6 a method has been described for performing PIFT, GIFT and LIFT simultaneously in a single test tube. Following standardizition of the calibration procedure of the flow cytometer and the preparation of the cell mixtures, tests can be measured with fixed flow cytometer settings allowing rapid measurements of large series of specimens and automated data analysis. The flow cytometric assay appeared more sensitive than the microscopic test.

In Chapter 7 we have compared the ELISA, the flow cytometric immunofluorescence tests and also a commercially available test based on solid phase red cell adherence (SPRCA) in microtiter plates. The flow cytometric immunofluorescence tests appeared comparatively most sensitive and the ELISA provides the second best assay with regard to sensitivity. Storage (for up to 6 months) of test cells for ELISA is possible. Preservation of test cells for immunofluorescence was not examined. SPRCA was relatively insensitive and storage of test cells was possible for up to 2 months. The lymphocytotoxicity-test (LCT) is often used as a standard test for the detection of HLA-antibodies. Both the ELISA and IFT are more sensitive than the LCT as regards the detection of HLA-antibodies.

In general, monitoring alloimmunization in patients receiving platelet transfusions requires the detection of alloantibodies including HLA-antibodies, platelet specific (HPA)-antibodies and ABH-antibodies. The lymphocytotoxicity test is the standard test for HLA-typing and frequently used to monitor HLA-alloimmunization. By using panels of frozen lymphocytes selected according to HLA-antigen composition, the specificity of the antibodies may be determined. By nature, the LCT cannot detect platelet specific alloantibodies. Therefore, tests using platelets as target cells such as an ELISA, must be included. The described combined PIFT and LIFT assays are valuable for the detection of both HLA-antibodies and platelet-specific antibodies as these tests are more sensitive for measuring

HLA-antibodies and at the same there may give additional information on antibody specificities. Limitations of both PIFT and ELISA are that anti-HPA-5a antibodies are not reliably detected; for this purpose an additional assay (e.g. MAIPA) would be required[29,30].

9.3 Platelet donor selection for refractory alloimmunized patients and the relevance of the platelet crossmatch

Soon after the introduction of platelet transfusion therapy the phenomenon of refractoriness to random donor platelets was recognized. The involvement of HLA-alloimmunization was first documented in 1965 by Bosch et al.[31] and HLA-matching of unrelated platelet donors for alloimmunized recipients was introduced soon thereafter[32-34]. Unfortunately, it became clear that a significant percentage of HLA-matched platelet transfusions administered to alloimmunized patients, did not result in satisfactory platelet increments. From the data presented in Table I it is apparent that 20-63% of the HLA-matched platelet transfusions are unsuccessful. To improve the donor selection procedure, lymphocyte or platelet crossmatching has been added to HLA-matching. A summary of the literature data on this subject is also presented in Table I.

To evaluate the value of procedures for selecting platelet donors for refractory patients, the patient population has to be characterized and the presence of non-immunological factors that are known to decrease post-transfusion platelet recoveries should be documented and taken into consideration in the analysis of the results. Unfortunately, there is no standard procedure for these investigations. On reviewing the literature, it is evident that the criteria used for evaluation of results such as platelet refractoriness, alloimmunization, successful platelet transfusion response and specification of non-immune factors, are highly variable from one report to another. The latter variability may contribute to the variability in the data and their interpretation. Generally, with regard to the LCT-crossmatch usually a high percentage of 20-40% of false negative test results i.e. a negative crossmatch associated with an unsuccessful transfusion outcome is seen[35-38,40]. Results with platelet crossmatch assays have been more successul. These tests use platelets as test cells and measure the amount of IgG that has

Table I. Summary of literature data on the additional value of platelet crossmatching for HLA-matched platelet transfusions in refractory patients

Author	Refr	Allo	NIF	Number Tx/pat	HLA-matching grade		Criterion Tx-success	% successful Tx			Assay (2nd antibody)
					A+BU	C+D		All Tx	Xm-neg	Xm-pos	
Filip, 1976	+	n.s.	-	53/10	n.s.	n.s.	24hr $PTPR_S$>20%	58	71	29	LCT
Herzig, 1977	n.s.	LCT	-	79/5	73	27	20hr CI>4.5	65	60	67	LCT
Brand, 1978	+	LCT	-	82/15	74	26	20hr CI>4.5	65	65	–	LCT
Brand, 1978	+	LCT	-	82/15	74	26	20hr CI>4.5	65	90	0	$PIFT_M$ (IgG)
Gmür, 1978	+	n.s.	-	51/24	33	n.s.	24hr CI>4.5	47	71	0	LCT
Tosato, 1980	+	n.s.	-	44/8	64	16	20hr CCI>7.5	55	82	37	LCT
Bowen, 1982	+	LCT	+	177/13	n.s.	n.s.	1-4hr PTPR>20%	80	90	17	LCT
Bowen, 1982	+	LCT	+	101/13	n.s.	n.s.	1-4hr PTPR>20%	74	84	60	$PIFT_M$ (IgG)
Bowen, 1982	+	LCT	+	78/13	n.s.	n.s.	1-4hr PTPR>20%	80	80	67	ELISA (IgG)
Ware, 1984	+	LCT	-	68/15	n.s.	62	1hr CCI>10	37	78	10	RIA(IgG)
Ware, 1984	+	LCT	-	68/15	n.s.	62	1hr CCI>10	37	72	16	$PIFT_M$ (IgG)
Yam, 1984	+	n.s.	-	38/n.s.	n.s.	71	1hr $PTPR_S$>20%	58	87	11	RIA(SPA)
Kickler, 1985	+	LCT	-	230/42	22	54	1hr CCI>7.5	66	92	27	RIA(IgG)
Kickler, 1985	+	LCT	-	230/42	22	54	20hr CCI>7.5	48	83	9	RIA(IgG)
Heal, 1987	+	ELISA	n.s.	222/51	14	64	1-4hr CCI>7.5	51	57	33	ELISA(IgG+IgM)
Millard, 1987	+	n.s.	+	51/7	n.s.	53	1hr CCI>7.5	61	89	11	ImmBead (IgG)
Millard, 1987	+	n.s.	+	51/7	n.s.	53	1hr CCI>7.5	61	83	29	RIA (IgG)
Brubaker, 1987	+	n.s.	-	51/6	n.s.	n.s.	1-2hr CCI>7.5	49	86	9	ELISA(IgG)
this thesis	+	LCT	-	82/30	n.s.	n.s.	1hr PTPR>20%	74	81	57	$PIFT_P$ (IgG)
this thesis	+	LCT	+	104/30	77	11	1hr PTPR>20%	66	75	45	$PIFT_P$ (IgG)

Table I (continued). List of abbrevations

Refr	: +: documented refractoriness to random multiple donor platelet transfusions
Allo	: presence of alloantibodies, assay used for detection specified
NIF	: non-immunological factors for platelet refractoriness: - : absent; + : included
Tx	: transfusion
Tx/pat	: no. of transfusions/no. of patient studied
Xm	: crossmatch
n.s.	: not specified

Assay: antibody test used for crossmatching
- LCT : lymphocytotoxicity test
- $PIFT_M$: platelet immunofluorescence test (microscopic evaluation)
- $PIFT_F$: platetet immunofluorescence test (flow cytometric evaluation)
- ELISA : enzyme-linked immunosorbent assay
- RIA : radio-immunoassay
- Immbead : Immunobead assay
- SPA : Staphylococcal Protein A test

Criterion Tx-success: formula used to calculate transfusion result and definition of successful transfusion
- CI = corrected increment
- CCI = corrected count increment
- PTPR = post transfusion platelet recovery
- $PTPR_S$ = PTPR, correction factor for splenic pooling included

$$CI = \frac{\text{platelet increment x b.s.a.}}{\text{no. of platelet units transfused}}$$

$$CCI = \frac{\text{platelet increment x b.s.a.}}{\text{no. of platelets transfused}}$$

$$PTPR = \frac{\text{platelet increment x blood volume}}{\text{no. of platelets transfused}} \times 100$$

$$PTPR_S = \frac{\text{platelet increment x blood volume}}{\text{no. of platelets transfused x 0.67}} \times 100$$

Platelet increment: posttransfusion - pretransfusion platelet count ($x10^9/L$)

b.s.a.: body surface area (m^2)

bound to the test platelets following incubation with patient serum (PIFT, ELISA, RIA). Nevertheless on the average 10-15% false negative crossmatches are still seen[37,41-43].

Our own experiences with a flow cytometric platelet crossmatch assay for donor selection for alloimunized patients have been described in Chapter 8. We observed that transfusions from HLA-matched donors with a negative platelet crossmatch gave results statistically significantly better than those with a positive platelet crossmatch. The frequency of false negative results in this series was 34 percent. Part of these false negative predictions may be explained by the presence of non-immunological adverse clinical factors, as will be discussed below in more detail, but even in the absence of these adverse factors still 19 percent of crossmatches was false negative. It is not clear why platelet crossmatching is still associated with so many false-negative crossmatches and how it might be improved. It is not due to ABO-incompatibility between donor and recipient as anti-A and anti-B antibodies are detected in the platelet crossmatch. Possibile explanations are, insufficient sensitivity of the assay for weak alloantibodies; the presence of drug-induced antibodies that remain undetected; incapability of the crossmatch test to detect some alloantibody-specificities or other causes not yet known.

ABO-incompatibility

ABO-incompatibility between donor and recipient may influence the platelet crossmatch and transfusion results as ABH-antigens are expressed on platelets[47-49]. An overall decrease of 23% of platelet increments was observed for ABO-incompatible platelets as compared with ABO-compatible platelets[50]. In many instances ABO-incompatibility between donor cells and recipient plasma does not lead to platelet transfusion failures. However, selected cases have been reported in which ABO-incompatibility between donor and recipient was associated with no platelet increment at all. In these instances very high IgG-anti-A,-B titers were observed[51]. In addition to the titer of the antibodies, the level of expression af ABH-antigens on platelets might be of importance. Recently, Ogasawara et al.[49] described that donors may be divided into two categories according to ABH-antigen expression i.e. a high-expression phenotype and a low-expression phenotype. Approximately

7% of donors belonged to the high-expression phenotype. Taken this variability in ABH-antigen expression and the variability in ABH-antibody titers into consideration, one may assume that platelets from a donor with the high-expression phenotype transfused into a patient with high antibody titers are particularly at risk of a poor post-transfusion platelet survival. The fact that the majority of recipients have low anti-A and anti-B IgG antibody titers and receive platelets from low expression donors may also explain why the majority of ABO-mismatched platelet transfusions are successful. Although it is clear that ABO-compatibility is of importance in some platelet transfusions, it is probably not an important cause for the false negative platelet crossmatches as assays using platelets as target cells may detect clinically relevant ABH-antibodies.

Non-immunological causes determining platelet transfusion results

In practice, many patients are exposed to one or more of non-immunological causes for reduced post-transfusion platelet survival. These factors may include e.g., fever, sepsis, splenomegaly, disseminated intravascular coagulation, bleeding[52-57]. Therefore, in our study we also analyzed platelet transfusions administered in the context of these non-immunological factors. Here, transfusions with a negative PIFT crossmatch gave still significantly better results than those with a positive PIFT crossmatch but only 50% of HLA-matched, platelet crossmatch negative transfusions were successful. Thus, another explanation for the reduced ability of a platelet crossmatch to reliably predict good transfusion responses is the presence of non-immunological causes for platelet refractoriness in the recipient. In another study it has been observed that the platelet crossmatch lost its predictive value in the presence of non-immunological adverse factors. One possible explanation for the discrepancy with our results is that a different platelet antibody test i.e. based on ^{125}I-labeled Staphylococcal Protein A, has been used[53].

These findings clearly underscore the important role that non-immunological causes play in platelet refractoriness. It can be foreseen that in the future non-immunological causes will be relatively more frequently the cause for platelet refractoriness as the use of leukocyte-depleted blood products will almost completely prevent primary HLA-alloimmunization.

9.4 A proposal for a platelet transfusion strategy

On the basis of the data obtained from the investigations reported here and the existing data from the literature, a strategy for platelet transfusions therapy in patients with thrombocytopenia due to decreased platelet production is proposed below. Evidently, this strategy does not apply to patients with thrombocytopenia due to increased platelet destruction or an altered platelet distribution.

Platelet transfusion strategy

Initial Phase

When thrombocytopenia is present or foreseen to develop, establish the HLA Class I Type of the patient, preferably before entering a period of thrombocytopenia. HLA typing may technically be difficult during leukocytopenia which usually accompanies thrombocytopenia due to bone marrow depression.

During severe thrombocytopenia, transfuse platelets to maintain platelet counts above $5\text{-}10 \times 10^9$/L to prevent bleeding, or at higher levels in case of bleeding or surgical intervention.

Blood Products

Use consistently leukocyte depleted platelet concentrates and red blood cell concentrates containing less than 5×10^6 leukocytes per transfusion.

Use - for adults - leukocyte depleted platelet concentrates with a guaranteed platelet content of at least 250×10^9 platelets even after a shelf life of 5 days.

Transfuse ABO-compatible platelet concentrates with regards to the ABO blood group of the platelets, whenever possible.

Evaluation of platelet transfusion

Evaluate the results of platelet transfusions by performing 1-hr and 16-20hr post-transfusion platelet counts. For untransfused male patients being at low risk for allo-immunization, one may consider to cancel 1hr counting as long as 16-20hr increments are sufficient.

Calculate exact post-transfusion recovery value to conclude if transfusion was successful or unsuccessful.

Unsuccessful platelet transfusion

Determine the cause of failure
- assess the presence of non-immunological causes for refractoriness
- examine the patients serum for the presence of alloantibodies

Use assays that detect both HLA-antibodies and non HLA platelet reactive antibodies e.g. PIFT+LIFT or LCT+ELISA.

In case of positive test, identify antibody specificities.

Transfusion regimen in case of unsuccessful platelet transfusions

• Alloantibodies absent
Continue transfusion with random donor platelets and increase platelet dose and frequency
- if not successful, apply HLA-matched transfusion as in vivo test.

• Alloantibodies present
Select platelet donors guided by the established antibody-specificity; if this is not clear, one may remember that 90% of antibodies are HLA-alloantibodies.

HLA-alloantibodies present:
Transfuse HLA-matched platelets, preferably with negative platelet crossmatch.
If possible, select for the first transfusion a donor who has HLA-match grade A or BU and who is ABO compatible with and negative platelet crossmatch for straightforward interpretation of results.
- if this is successful: continue HLA-matched transfusions and if necessary, because of donor unavailability, change to ABO-incompatible donors and/or different HLA-match grades
- if this is not successful, consider:
  - ABH-antibodies (determine IgG anti-A/-B-titers in serum recipient)
  - platelet specific antibodies: if assays detecting PB-IgG are negative, also perform assay detecting anti-HPA-5b antibodies e.g. MAIPA test.

HPA-alloantibodies present:
- select HPA-compatible platelet donors from platelet specific antigen typed donor file.

High titer IgG-ABH-antibodies present:
- select ABO-compatible donors.

Combination of alloantibodies present:
- select donors accordingly.

• Platelet autoantibodies present (in patients with decreased platelet production)
- take into consideration the absence or presence of alloantibodies, as described above
- if not succesful: increase platelet dose
- if still not successful: consider additional treatment of the patient e.g., high dose immunoglobulins intravenously.

• When non-immunological causes for platelet refractoriness are present
- take into consideration the absence or presence of alloantibodies, as described above
- if not successful: increase platelet dose.

• If no HLA-compatible (match grade A or BU) donor is available
- match on cross-reactive HLA-antigens
- match on serologically acceptable mismatches i.e. HLA-antigens to which no antibodies are present in the patients serum
- consider experimental approaches.

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Definitions

Platelet refractoriness
- two consecutive, unsuccessful random donor platelet transfusions.

Successful platelet transfusion
- One-hr post-transfusion platelet recovery greater than 20%.

HLA-match grade
- A : all 4 antigens (HLA-A and HLA-B locus) in donor identical to those of recipient
- BU: only 2 or 3 antigens detected in donor, all present in recipient.

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SAMENVATTING

Bloedplaatjes zijn kleine celfragmenten in het bloed die een belangrijke functie bij de bloedstolling vervullen. Zij worden in het beenmerg geproduceerd. Een tekort aan bloedplaatjes treedt onder andere op wanneer het beenmerg onvoldoende produceert. Een voorbeeld van zo'n situatie is een patient met kanker die met chemotherapie wordt behandeld. Als bijwerking van deze behandeling wordt de aanmaak van bloedcellen - en dus ook die van de bloedplaatjes - in het beenmerg tijdelijk onderdrukt. Soms resulteert dit in een sterke verlaging van het aantal bloedplaatjes. Door het tekort aan bloedplaatjes in het bloed heeft de patient een verhoogde bloedingsneiging en kan daardoor spontaan bloedingen krijgen. Deze bloedingen kunnen qua ernst varieren van licht (b.v. huidbloedingen) tot zeer ernstig en levensbedreigend (b.v. hersenbloeding).

Om dit - veelal tijdelijke - tekort aan bloedplaatjes op te vangen zijn transfusie's met (donor)bloedplaatjes belangrijk. Door 2 à 3 maal per week een transfusie van bloedplaatjes toe te dienen kunnen ernstige bloedingen meestal worden voorkomen.

Er is echter een belangrijk probleem bij patienten die regelmatig bloedplaatjestransfusies nodig hebben. Na verloop van tijd kan de patient afweerstoffen (= antistoffen) tegen donorbloedplaatjes gaan vormen, die het resultaat van de transfusie belemmeren. Dat er antistoffen worden gevormd is het gevolg van het feit dat er bloedgroepen op de bloedplaatjes voorkomen die veelal verschillend zijn van mens tot mens. Wanneer een patient een transfusie krijgt van een donor bij wie de bloedgroepen van de bloedplaatjes anders zijn dan die van de patient, dan kan de patient deze bloedplaatjes als vreemd herkennen en als gevolg tegen deze bloedgroepen antistoffen gaan maken. De bloedgroepen die hierbij van belang zijn, zijn vooral de humane leukocyten (HLA) antigenen. Deze komen ook op de witte bloedcellen (= leukocyten) voor. Andere kenmerken op bloedplaatjes zijn de "gewone" bloedgroepen van het ABO-systeem en tenslotte zijn er nog kenmerken die uitsluitend op de bloedplaatjes voorkomen, de zogenaamde plaatjes-specifieke bloedgroepen.

Al snel na de ontwikkeling van bloedplaatjestransfusies werd dit probleem van antistofvorming, alloimmunisatie genaamd, herkend. Alloimmunisatie gericht tegen bloedplaatjes is een ernstige complicatie bij patienten die bloedplaatjestransfusies nodig hebben. Bij meer dan de helft van de patienten werd alloimmunisatie na kortere of langere behandelingsduur waargenomen. Het effekt van deze antistoffen is dat getransfundeerde donorbloedplaatjes zeer snel, meestal binnen een uur, worden afgebroken zodat de transfusie onwerkzaam is. Hierdoor kan de patient niet afdoende tegen bloedingen worden beschermd bij een verlaging van het aantal bloedplaatjes in het bloed.

In dit proefschrift wordt onderzoek beschreven betreffende de problematiek van de antistofvorming (de alloimmunisatie) bij bloedplaatjestransfusies. Onderzoek is verricht met betrekking tot:
- het voorkómen van alloimmunisatie
- het aantonen van antistoffen gericht tegen bloedplaatjes
- de selectie van bloedplaatjesdonoren voor patienten met antistoffen.

In **Hoofdstuk 1** wordt een overzicht gegeven van de geschiedenis op het gebied van trombocytentransfusies en alloimmunisatie. Uit onderzoek is duidelijk geworden dat bij transfusie van bloedplaatjes het niet zozeer de bloedplaatjes zelf zijn die antistoffen opwekken. Het zijn vooral de witte bloedcellen (de leukocyten) die als "verontreiniging" in de verschillende bloedprodukten aanwezig zijn waartegen antilichamen worden gemaakt. Wanneer bij de transfusie-bewerking de leukocyten zoveel mogelijk uit de bloedplaatjestransfusies - en ook uit de rode bloedceltransfusies - worden verwijderd, treedt deze antistofvorming niet meer op. Dit gebeurt dan alleen nog bij uitzondering namelijk bij die patienten die eerder in contact met "vreemde" leukocyten zijn geweest. Dit kan bijvoorbeeld het geval zijn geweest bij een eerdere bloedtransfusie (zoals bij een operatie). Bovendien hebben ongeveer 40% van vrouwen die 1 of 2 maal zwanger zijn geweest dergelijke antistoffen gevormd door contact met het bloed van het kind tijdens de zwangerschap. Bij deze groep patienten zijn de antistoffen in de loop der jaren verdwenen. Wanneer echter later weer bloedplaatjestransfusies worden toegediend blijkt ongeveer 40% van deze vrouwen na transfusie weer

opnieuw antistoffen te vormen; dit wordt de secundaire immuunrespons genoemd.

Tot nu toe waren met betrekking tot deze secundaire immuunrespons alleen gegevens beschikbaar van transfusies van bloedprodukten die gemiddeld toch nog 20-50x10^6 leukocyten per transfusie bevatten. In **Hoofdstuk 2** is een onderzoek beschreven waarin is nagegaan of een verdergaande verwijdering van leukocyten secundaire HLA-alloimmunisatie wel zou kunnen voorkomen. Hiertoe werden bloedplaatjestransfusies en rode bloedcelconcentraten bereid die ten hoogste 5x10^6 leukocyten bevatten. Deze gezuiverde produkten werden getransfundeerd bij vrouwen die eerder zwanger waren geweest. Een tweede groep patienten, eveneens vrouwen met eerdere zwangerschappen, ontving ter vergelijking standaard bloedplaatjesconcentraten (=leukocyten bevattend). Nagegaan werd hoe de antistofvorming in beide groepen verliep en hoe de effectiviteit van de bloedplaatjestransfusies was. De resultaten van dit onderzoek toonden aan dat ook met deze "verbeterde" produkten de secundaire HLA-alloimmunisatie niet kon worden voorkomen. Voor de toekomst betekent dit dat onderzocht moet worden of transfusies met nog minder leukocyten wellicht alsnog het gewenste effekt kunnen hebben. Helaas zijn filters waarmee dergelijke produkten gemaakt zouden kunnen worden, nog niet beschikbaar. Een geheel andere verklaring zou kunnen zijn dat in dit speciale geval niet de leukocyten maar de trombocyten zelf de antistofvorming opwekken. Dan zal de methode van leukocytenverwijdering bij deze groep patienten dus nooit effectief kunnen zijn.

De leukocyten worden meestal uit de bloedprodukten verwijderd door speciale bloedfilters te gebruiken. Hoewel op de wijze meer dan 99% van de leukocyten kunnen worden verwijderd, blijven er toch nog ongeveer 2x10^6 in het bloedplaatjesconcentraat achter. In **Hoofdstuk 3** is een vergelijkend onderzoek naar de eigenschappen van 4 verschillende leukocyten-filters uitgevoerd. Het betrof de volgende typen filters: Pall PL50S, Pall PL100S, Sepacell PL-5N en Sepacell PL10N. Alle geteste filtertypen verwijderden tussen de 99% en 99,9% van de aanwezige leukocyten. Bij gebruik van het Pall-PL50S filter was het bloedplaatjesverlies door de filtratieprocedure 7%. Dit resultaat was beter dan dat van de andere filters waarbij het verlies aan

bloedplaatjes gemiddeld 11% was.

Het is belangrijk om te beschikken over een laboratoriumtest waarmee nagegaan kan worden of zich in het bloed van een patient antistoffen tegen bloedplaatjes bevinden. Omdat de aanwezigheid van antistoffen slechts een van de mogelijke verklaringen is voor een slecht transfusieresultaat, kan een dergelijke test behulpzaam zijn om de oorzaak vast te stellen van een slecht transfusieresultaat. Een andere toepassing van zo'n test is om voor een patient met antistoffen passende donoren te selecteren.
Een aantal technieken voor het aantonen van antistoffen tegen bloedplaatjes is in dit proefschrift beschreven. Zo'n test dient kwalitatief aan hoge eisen te voldoen. Een goede test dient in de eerste plaats gevoelig te zijn. Dit houdt in dat ook zwakke antistoffen met de test kunnen worden aangetoond. In de tweede plaats dient de test ook specifiek te zijn. Dit betekent dat er echt geen trombocytenantistoffen zijn als de test negatief is.
In **Hoofdstuk 4** is de zgn ^{51}Cr-lysis test met bloedplaatjes beschreven. Met deze test konden antistoffen tegen trombocyten worden aangetoond. Een nadeel is dat bepaalde typen antistoffen (de niet-complement bindende antistoffen) niet kunnen worden aangetoond en dat er bij de test gebruik wordt gemaakt van radioactiviteit.
In **Hoofdstuk 5** wordt als verdere ontwikkeling een ELISA-test beschreven die bovengenoemde nadelen niet bezit en die bovendien veel sneller is. Deze test kan ook met ingevroren testcellen worden uitgevoerd. Dit heeft als voordeel dat de donor niet voor iedere bepaling naar het laboratorium hoeft te komen. De resultaten van deze test bleken even goed uit te vallen als die van de tot dan toe als standaard gehanteerde plaatjesimmunofluorescentie test.
In **Hoofdstuk 6** is een testmethode beschreven waarmee naast antistoffen tegen bloedplaatjes gelijktijdig antistoffen tegen leukocyten - granulocyten en lymfocyten - kunnen worden aangetoond. Hierbij werd gebruik gemaakt van immunofluorescentie en analyse met een flow cytometer. Met een dergelijk apparaat kunnen de verschillende celtypen i.c. bloedplaatjes, granulocyten en lymfocyten in één monster herkend worden en kan de antistofbelading per celtype worden vastgesteld. Deze test bleek zeer gevoelig maar wel arbeidsintensief.

Naast deze in ons laboratorium ontwikkelde testen zijn ook door commerciele firma's testen ontwikkeld. Een van deze testen, de "solid phase red cell adherence (SPRCA)" test, is in **Hoofdstuk 7** vergeleken met de hierboven genoemde ELISA en flow cytometrische immunofluorescentie testen. De flow cytometrische immunofluorescentietesten bleken het meest gevoelig. De SPRCA was 't minst gevoelig.

In **Hoofstuk 8** is het gebruik van de flow cytometrische plaatjes-immunofluorescentie test (PIFT) onderzocht als een methode om bloedplaatjesdonoren te selecteren voor patienten met antistoffen. Bij 104 transfusies werd met deze techniek een monster van de transfusie getest met een bloedmonster van de patient. Wanneer de PIFT negatief was (d.w.z. geen antistoffen aangetoond) dan waren de transfusieresultaten beter dan wanneer de PIFT positief (d.w.z. wel antistoffen aangetoond) was. Door bij elke transfusie een PIFT-test te doen zou in theorie het transfusieresultaat kunnen worden voorspeld. In de praktijk blijkt de PIFT hiervoor een te gecompliceerde test. Daarom zal zeker verder onderzoek gedaan worden naar weer nieuwere testen die de kwaliteiten van de PIFT behouden maar die wel sneller en eenvoudiger zijn.

In **Hoofdstuk 9** tenslotte is een algemene discussie over de bevindingen in dit proefschrift opgenomen tesamen met een samenvatting van de verschillende hoofdstukken. Tevens werd toegevoegd een voorstel om volgens een eenduidige werkwijze het beleid voor het geven van bloedplaatjestransfusies voor een bepaalde patient te bepalen.

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CURRICULUM VITAE

Kees Sintnicolaas werd geboren op 30 april 1951 te Bolnes. In 1969 werd het diploma Gymnasium-ß behaald aan het Marnix Gymnasium te Rotterdam. In 1970 werd - na eerst een jaar piano gestudeerd te hebben aan het Rotterdams Conservatorium - de studie geneeskunde aangevangen aan de Medische Faculteit te Rotterdam. Het artsexamen werd in 1977 behaald. Direct aansluitend werd hij werkzaam in de dr Daniel den Hoed Kliniek te Rotterdam in het kader van een onderzoeksprojekt "Hematologische supportive care" (projectleiders dr H.M. Vriesendorp en - toen nog - dr B. Löwenberg) binnen de Afdeling Hematologie (Hoofd Mw W. Sizoo). In 1986 werd hij in hetzelfde ziekenhuis aangesteld als Hoofd Bloedtransfusiedienst binnen de afdeling Klinische Chemie (Hoofd dr W.G. Haije). In 1993 trad hij als transfusiearts in dienst van de Stichting Rode Kruis Bloedbank Rotterdam (Medisch Directeur dr D.J. van Rhenen) met als aandachtsgebied het opzetten van een laboratorium voor HLA-typering.