Platelet Transfusion Therapy.
Optimal Donor Selection With a Combination of Lymphocytotoxicity and Platelet Fluorescence Tests

By A. Brand, A. van Leeuwen, J. G. Eernisse, and J. J. van Rood

Although the value of HLA matching for the selection of platelet donors for patients refractory to random platelets is beyond doubt, even perfectly matched combinations sometimes fail to give a satisfactory transfusion response. With HLA typing and negative lymphocytotoxicity crossmatches, 35% of the platelet transfusions administered to 15 patients gave disappointing results (29 of 82). Additional crossmatching with the newly developed platelet fluorescence test described in this paper reduced the unexpected transfusion failures to 7% (6 of 82). Five of these failures were observed in one patient. The target of the antibodies detected with this platelet fluorescence test is not yet fully specified. It seems probable that both HLA and platelet-specific non-HLA antibodies were detected. No correlation of the results of platelet transfusions with the presence or absence of leukoagglutinating antibodies was found.

Platelet transfusions from random donors are ineffective in alloimmunized recipients. Antibodies against HLA antigens, which platelets have in common with leukocytes and other cells, are probably the main cause of this refractoriness. Bosch et al. showed in 1965 that platelets from HLA-identical relatives could survive normally in severely sensitized patients. This finding was confirmed by others and later extended to the successful use of unrelated HLA-compatible donors.

There are several ways to obtain suitable platelet donors for immunized patients, most of them based on HLA typing of lymphocytes. Donors identical for HLA antigens, with closely-related antigens, or with minimal mismatches in relation to the recipient can be selected from relatives or from a file of HLA-typed donors. Centers without typing facilities sometimes perform only lymphocytotoxicity crossmatches between the serum of the recipient and lymphocytes of the family donors or volunteers.

However, 15%—40% of multitransfused patients fail to make detectable leukocyte antibodies (the so-called low responders) but are nevertheless refractory to random platelets, showing that platelets are an extremely sensitive target for (still undetectable (HLA?) antibodies. On the other hand, the lymphocytotoxicity test may not offer the right approach to the problem because we and others have seen cases in which transfusion of HLA-identical platelets resulted in a poor platelet increment that could not be ascribed to clinical causes such as defibrination syndromes or septicemia. This negative
result is thought to be due to antibodies directed against ill-defined HLA antigens (e.g., from the HLA-C series) or platelet-specific antigens,\textsuperscript{12,15} or, according to Herzig et al., to an innocent bystander mechanism.\textsuperscript{14} The last seems unlikely in our study because we routinely infused almost pure platelet preparations with hardly any other cell type to cause bystander destruction. Because the preparation of matched platelet transfusions is time-consuming and expensive, a reliable platelet crossmatch would be most welcome.

This paper reports the results obtained with a method using immunofluorescence for crossmatching platelets, permitting a correct prediction of the effect of platelet transfusion in more than 90\% of patients refractory to random platelets.

**MATERIALS AND METHODS**

Fifteen patients were studied. All were thrombocytopenic due to bone marrow insufficiency and had a history of extensive transfusion therapy with red cells, platelets from random donors, and in some cases granulocytes. When two or three successive random platelet transfusions (pooled platelets from 4 U blood) failed to give the expected posttransfusion increment at 1 hr or about 20 hr, the patient was considered to be alloimmunized. This situation occurred in all 15 patients. Patients with septicemia or disseminated intravascular coagulation were excluded from this study.

*HLA typing* was done with the two-step complement-dependent microlymphocytotoxicity test (CDC test).\textsuperscript{16} The antisera used were directed against the following antigens: A1, A2, A3, A9, A11, AW25, AW26, A28, A29, AW30, AW31, AW32, AW33, AW34, AW36; B5, B7, B8, B12, B13, B14, BW15.1, BW15.2, BW16, BW17, B18, BW21.1, BW21.2, BW22, B27, BW35, BW37, BW38, BW39, BW40, BW41, BW42, BHR, BTI, B407; CW1, CW2, CW3, CW4; W4, W6.

*Donor selection.* Patients' serum samples were screened at regular intervals for lymphocytotoxic antibodies against samples from a panel of more than 50 donors. If possible, the specificity of the antibodies was determined and taken into account for donor selection. In all cases family studies were performed to establish the genotype, and if there was no contraindication (for instance, if bone marrow transplantation was contemplated) relatives were included as donors. If relatives could not be used, donors were selected from about 4000 HLA-typed donors of the Red Cross Blood Transfusion Service, about 100 of whom were probably or proven homozygous for HLA-A and -B antigens. Incompatibility for ABO was not considered a contraindication for use of a donor.

*HLA matching.* Different degrees of matching were distinguished:

- **A match:** identity for all HLA-A and -B antigens or compatibility if homozygosity of the donor had been proved.
- **B match:** no compatible antigens in the donor, but only two or three HLA antigens detectable on the donor cells without proven homozygosity.
- **C, match:** one antigen of the donor incompatible with the patient’s HLA type.
- **C2 match:** as C, but two antigens incompatible.

Examples: Patient A2 A3 B7 BW14

Donor A2 A3 B7 BW14 (A match)
A3 A3 B7 B7 (A match)
A2 A3 B7 (B match)
A2 A3 B7 B12 (C, match)
A2 AW32 B7 B27 (C2 match)

For one patient (No. 8) with a very unusual HLA type, three donors with only one antigen matching the patient’s (C, match) were included. Because only four antigens within the HLA-C system could be recognized, incompatibilities within this system for establishing the match grade were disregarded. This decision was considered permissible because of the strong linkage disequilibrium between HLA-B and -C antigens.
Crossmatching. Donor cells were crossmatched with the patient’s serum before transfusion. The following techniques were used:

1. Complement-dependent cytotoxicity (CDC): a two-step procedure.\textsuperscript{16}
2. Leukoagglutination test: ethylenediaminetetraacetate (EDTA) blood was used in a macro-agglutination technique;\textsuperscript{10,17,18} this was performed only in some donor-recipient combinations.
3. Platelet immunofluorescence (PIF) test: From 9 ml of donor blood collected in 1 ml 5\% EDTA in saline, platelets were isolated by differential centrifugation, washed three times in 10 ml 0.3\% EDTA in phosphate-buffered saline (PBS) (washing solution), and resuspended in the same solution to a concentration of $5 \times 10^7$ platelets/ml. All centrifugations and incubations were done at $20^\circ - 22^\circ$C. One drop of this platelet suspension and one drop of inactivated patient’s serum were incubated for 60 min, after which the platelets were washed three times in about 3 ml of the washing solution. After complete removal of the supernatant, two drops of a rhodamine-conjugated antiglobulin serum [GAHu/IgG(Fc) TRITC: Nordic, Tilburg, The Netherlands; before use, the antiserum was diluted 1:7 with saline] were added and the mixture incubated for 30 min. The platelets were then washed again three times in about 3 ml of washing solution and the platelet pellet resuspended in one drop of the 0.3\% EDTA in saline, transferred to a microscope slide, covered with a coverglass, and sealed with paraffin. Reading was done with an ordinary Zeiss microscope with an HBO 50-W lamp, filter combination LP515-SP560, and barrier filter LP590. The number of fluorescent platelets per 200 platelets was established. After complete removal of the supernatant, two drops of a rhodamine-conjugated antiglobulin serum [GAHu/IgG(Fc) TRITC: Nordic, Tilburg, The Netherlands; before use, the antiserum was diluted 1:7 with saline] were added and the mixture incubated for 30 min. The platelets were then washed again three times in about 3 ml of washing solution and the platelet pellet resuspended in one drop of the 0.3\% EDTA in saline, transferred to a microscope slide, covered with a coverglass, and sealed with paraffin. Reading was done with an ordinary Zeiss microscope with an HBO 50-W lamp, filter combination LP515-SP560, and barrier filter LP590. The number of fluorescent platelets per 200 platelets was established. As a control, donor platelets were incubated with autologous or AB serum, which gave a percentage of fluorescent platelets of about 3\% (range 0\%, 10\%). The net percentage of fluorescent platelets (donor platelets with patient’s serum minus donor platelets with autologous serum) was calculated.

Platelet transfusions. Platelet substitution was usually given when the platelet count had dropped to 5000/cu mm or to a higher level at which the individual patient was known to show bleeding symptoms (epistaxis, hematuria). Platelets from 3 U blood [440 ml blood, 60 ml acid citrate dextrose (ACD) solution (R/ACD: disodium citrate 3.3: dextrose 3.3: distilled water ad 100)] were collected by conventional plateletpheresis and processed with extra ACD added to the platelet-rich plasma. All centrifugations were carried out at $22^\circ$C. The combined resuspended sediments (150 - 200 ml) were subjected to an extra centrifugation step of 5 - 7 min at 400 g. The sediment containing most of the white cells was discarded. This process is a routine procedure in our laboratory and is intended to prevent immunization and avoid febrile reactions. Approximately 25\% of the platelets are lost in this procedure for the preparation of leukocyte-poor platelet suspensions, which then contain fewer than $10^7$ white cells. After a final centrifugation (20 min, 1100 g, $22^\circ$C) the platelet concentrate was resuspended in 10 - 20 ml plasma and contained an average of $2 - 2.5 \times 10^11$ platelets. Platelet counting was performed according to Feissly and Lüdin.\textsuperscript{19} Effectiveness of the transfusion was judged from the posttransfusion increment, calculated as:

\[
\text{Effectiveness} = \frac{(\text{posttransfusion platelet count} - \text{pretransfusion platelet count}) \times \text{BSA}}{\text{number of units transfused}}
\]

with BSA (body surface area) in sq m, estimated from height and weight.\textsuperscript{20} This increment was determined 1 hr and about 20 hr after transfusion. For a positive transfusion effect, the platelet increment at these times had to be $>7500$ and $>4500$ platelets/cu mm, respectively, and had to be maintained above the pretransfusion level for about 3 days.

RESULTS

All of the 15 patients had lymphocytotoxic antibodies against the panel in a wide range (8\% - 98\%) (Table 1). Sixty-nine donors were selected for these 15 patients, providing 82 donor-recipient combinations; 18 of the 69 donors were homozygous for HLA-A and -B. The degree of matching is listed in Table 1. The crossmatch between the serum of the recipient and the lymphocytes of the corresponding platelet donor in the CDC test was always negative. Only 7 pa-
Patients' sera were tested for leukoagglutinating antibodies with the prospective donor in 34 combinations because it soon became evident that the results of this test showed no correlation with the transfusion results in vivo. The test was positive in six combinations, of which four were associated with good results in vivo.

Table 1. Relevant Data on the 15 Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex, Age (yr)</th>
<th>Diagnosis</th>
<th>HLA Type</th>
<th>Donors' Match Grade</th>
<th>Patients' Score (CDC (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 23 AA</td>
<td>A2, A3, B7, BW14, W6</td>
<td>A B C1 C2 C3</td>
<td>2 5 2 1</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>M, 14 AA</td>
<td>A1, A3, B8, W6</td>
<td>A B C1 C2 C3</td>
<td>6 7 1</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>M, 31 AML</td>
<td>A2, A2, B12, B29, CW4, W4</td>
<td>A B C1 C2 C3</td>
<td>2 1</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>M, 42 AML</td>
<td>A1, A1, B8, W6</td>
<td>A B C1 C2 C3</td>
<td>7 3</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>F, 45 AML</td>
<td>A1, A3, B7, W6</td>
<td>A B C1 C2 C3</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>F, 56 AML</td>
<td>A2, A9, BW15, C, CW3, W6</td>
<td>A B C1 C2 C3</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>7</td>
<td>M, 20 AA</td>
<td>A2, A9, B8, BW5, CW4, W6</td>
<td>A B C1 C2 C3</td>
<td>2 2 2</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>M, 8 AA</td>
<td>AW30, AW33, B14, B18, W6</td>
<td>A B C1 C2 C3</td>
<td>1 2 3</td>
<td>96</td>
</tr>
<tr>
<td>9</td>
<td>F, 12 AA</td>
<td>A2, A3, B7, W6</td>
<td>A B C1 C2 C3</td>
<td>2 1</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>M, 74 AA</td>
<td>A2, AW40, B12, B12, CW3, W4, W6</td>
<td>A B C1 C2 C3</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>F, 45 AA</td>
<td>A1, AW30, B8, B14</td>
<td>A B C1 C2 C3</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>12</td>
<td>M, 47 AA</td>
<td>A3, AW30, B8, B18</td>
<td>A B C1 C2 C3</td>
<td>1 2</td>
<td>25</td>
</tr>
<tr>
<td>13</td>
<td>M, 63 AA</td>
<td>A3, AW26, B7, BW16, W4, W6</td>
<td>A B C1 C2 C3</td>
<td>2 1 1</td>
<td>65</td>
</tr>
<tr>
<td>14</td>
<td>F, 44 AML</td>
<td>A2, AW29, B7, BW13, CW3, W6</td>
<td>A B C1 C2 C3</td>
<td>7</td>
<td>98</td>
</tr>
<tr>
<td>15</td>
<td>M, 25 AA</td>
<td>A2, A3, B13, BW35, CW4</td>
<td>A B C1 C2 C3</td>
<td>2 2 1</td>
<td>20</td>
</tr>
</tbody>
</table>

Totals 31 30 8 10 3

AA, aplastic anemia; AML, acute myeloid leukemia.

*Number of donors of the various match grades used for platelet transfusions.

†Percentage of samples from lymphocyte panel positive with a patient's serum.

Fig. 1. Percentage of fluorescent platelets in the various PIF tests and the results of their administration. A, total number of transfusions; B, number of unsuccessful transfusions.
Table 2. Results of Platelet Transfusions in Relation to the Platelet Immunofluorescence Test

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>Good</th>
<th>Bad</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 15%</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>&lt; 15%</td>
<td>53</td>
<td>6</td>
</tr>
</tbody>
</table>

In all instances the CDC test was negative.

**Platelet Immunofluorescence Test (PIF)**

The results of this test in the various donor-recipient combinations are shown together with the survival of platelets from the same combinations in Fig. 1. From the graph it can be seen that platelet survival was not normal in any of the combinations with more than 15%, fluorescence, and on the basis of these findings it was concluded that fluorescence > 15%, had to be considered positive.

In Table 2 these results are tabulated, showing that 23 of the 29 failing combinations were correctly predicted by the PIF test, leaving six false-negative tests. The overall percentage of false predictions by the CDC test was 35%, compared with 7% of the PIF test. No false positive results were observed in either test.

The 20-hr recovery values for the different match grades in association with the PIF test are shown in Fig. 2. In all of the fluorescent-positive combinations the 20-hr increment was less than 4500/cu mm/sq m BSA/U transfused. The six combinations without a posttransfusion increment despite a negative PIF crossmatch are indicated by arrows.

It is noteworthy that positive PIF tests also occurred in HLA-identical and -compatible combinations (A and B matches), even in three of the seven HLA-identical sibling combinations, and that the six PIF failures occurred in only two patients.

![Fig. 2. Platelet increment 20 hr after transfusion of fluorescence-positive (·) and negative (o) platelets of the various match grades.](image-url)
Because these test failures were restricted to HLA-nonidentical combinations (C matches), the sensitivity of this test to detect HLA antibodies was compared with that of the CDC test on lymphocytes. For this comparison monospecific anti-HLA typing sera were tested in serial dilutions against samples from our panel donors with the lymphocytes in the CDC tests and against the platelets from the same individuals in the PIF test.* We found that both tests could miss weak (highly diluted) anti-HLA antibodies.

DISCUSSION

The fluorescence test described here was technically quite simple, and in most of our cases discrimination between positive and negative did not pose any problems. However, a few tests fell in the range 10° - 20°. The fact that in our material four combinations with 20°, 20°, 19°, and 17° positive cells gave a bad transfusion result and two with 11° and 14° a good result may have been a matter of chance.

More experience will show whether or not an exact cutoff point can be established. In six combinations the PIF test was negative but the transfusion result showed incompatibility. One of these failures occurred in patient No. 2. The donor (C2 match) was his haploidentical father who also donated bone marrow, the infusion of which was followed by a febrile reaction. The lymphocytotoxicity, leukoagglutination, B cell lymphocytotoxicity, and lymphocyte fluorescence tests and the PIF test remained negative on several occasions. Only the mixed lymphocyte culture (MLC) and cell-mediated lympholysis (CML) tests were consistently positive as expected. Theoretically, this failure could have been caused by cellular immunity rather than by some type of antibody active against the antigens from his father.

The other five failures all occurred in one patient, No. 8, in HLA-incompatible combinations (two C2, three C3). Although the patient's serum contained leukocyte antibodies against 96° of panel samples, it was negative with these five donors. The PIF test can also fail to demonstrate weak HLA antibodies, and it seems likely therefore that these transfusion failures were due to weak anti-HLA antibodies not detectable by either the lymphocytotoxicity or the PIF tests. More sensitive techniques, such as the antibody consumption test and the LDA (lymphocyte dependent antibody) test, will in the future be applied to find out if (relative) insensitivity is really involved here.

Although the antibodies that can be detected with this technique are of great importance in donor selection for platelet transfusion therapy, it is not yet clear towards which antigens they are directed. Some of the positive platelet crossmatches in the HLA-incompatible combinations may be due to low-titered or non-C' fixing HLA antibodies.

For the HLA-compatible combinations the positive crossmatches are more difficult to explain. HLA-C is not very likely, because it segregates with HLA-B.

*This technique, like the C' fixation test on platelets,21,22 provides an indication that in some of the individuals with HLA-B antigens 8 and 12, these antigens cannot be demonstrated on platelets. Since this might also be the case for B15, B17, and B18, a more extensive study on this point is in progress.
moreover, we have the impression that antigens of this region, to the extent that they have been defined, are better demonstrated on lymphocytes, which would have meant a positive crossmatch in the CDC test.

Another possible explanation is that these positive tests are caused by antibodies against platelet-specific antigens. This result was probable in patient No. 6, who showed a positive fluorescence test with 100% of panel samples and a negative one with an established Zw$^b$ individual, possibly indicating the presence of an anti-Zw$^a$ antibody. We hesitate to explain all other unexpected positive PIF tests in this way. The percentage of positive tests that can be expected in case of an antibody against one of the four known platelet-specific antigen systems—Zw, PL$^5$, Ko, Duzo$^a$—ranges from 15% to 99%. Because all our patients also had anti-HLA antibodies, we could take into account only the percentage of positive PIF tests obtained in HLA-compatible combinations. In the four patients for whom a substantial number of such donors could be found, the frequency of a positive PIF test was compared with the frequencies of the established platelet-specific antigens. The results indicate the presence of antibodies against known Ko$^a$, Zw$^a$, and Zw$^b$ (and also unknown!) specificities, but the numbers are too small for reliable frequency estimation, and the specific antisera originally used for the description of these antigens were not available for comparison. Firm conclusions are therefore not justified. Studies on the presence of platelet-specific antibodies in these sera after absorption of HLA-antibodies with lymphocytes are in progress.

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