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, $430 \times 10^6$ per transfusion) or leukocyte-depleted random single-donor platelet transfusions. Leukocyte depletion to less than $5 \times 10^6$ leukocytes per platelet transfusion (mean leukocytes, $2 \times 10^6$ 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 $5 \times 10^6$ per transfusion does not reduce the incidence of refractoriness to random donor platelet transfusions because of boosting of anti-HLA antibodies.

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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 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, especially in patients with previous pregnancies.

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 Productkwaliteitsbureau voor Bloedtransfusieapparatuur en Infusievloeistoffen BV, Emmen-Compascuum, The Netherlands) in a closed system using a sterile docking device (SCD: Haemonetics, Braintree, MA). This procedure results in a median number of $0.4 \times 10^6$ leukocytes/U with 99% of units containing less than $5 \times 10^6$ leukocytes.
PREVENTION OF SECONDARY HLA-ALLOIMMUNIZATION

Table 1. Patient Characteristics

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

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 prostacyclin to the platelet suspension before filtration. 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 predilution 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 ≈ 5 x 10^6/L leukocytes, which corresponds to ≈ 2 x 10^9 leukocytes per platelet transfusion.

Transfusion regimen. ABO-compatible platelets were transfused prophylactically when the platelet count dropped below 10 to 20 x 10^9/L, or therapeutically for invasive procedures and in case of major hemorrhagic symptoms at platelet counts above 10 to 20 x 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) x 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 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-Meier. 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 of 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 preceding 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

<table>
<thead>
<tr>
<th></th>
<th>Standard Group</th>
<th>Filtered Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of products</td>
<td>347</td>
<td>324</td>
</tr>
<tr>
<td>No. of platelets (×10^9)</td>
<td>352 ± 94</td>
<td>302 ± 102</td>
</tr>
<tr>
<td>No. of leukocytes (×10^8)</td>
<td>430 ± 703</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>No. of product with leukocytes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 x 10^6</td>
<td>0 (0%)**</td>
<td>159 (62%)</td>
</tr>
<tr>
<td>2.5 x 10^6</td>
<td>3 (1%)</td>
<td>133 (44%)</td>
</tr>
<tr>
<td>6-10 x 10^6</td>
<td>1 (3%)</td>
<td>7 (2%)</td>
</tr>
<tr>
<td>11-20 x 10^6</td>
<td>7 (2%)</td>
<td>3 (1%)</td>
</tr>
<tr>
<td>&gt;20 x 10^6</td>
<td>317 (97%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>ND</td>
<td>18</td>
<td>21</td>
</tr>
</tbody>
</table>

No. of platelets and leukocytes are expressed as mean ± SD. Values in parentheses represent the percentage of counted products. Abbreviation: ND, not determined.
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 $5 \times 10^6$ 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 25% reduction in the actuarial probability of becoming refractory (95% CL: $-21\%$, $+31\%$).

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.

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 remaining 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 $5 \times 10^6$ leukocytes per transfusion does not reduce the incidence of platelet refractoriness and anti-HLA antibodies. In about 40% of the patients studied, anti-HLA alloantibodies developed irrespective of whether platelet products were 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. Secondly, it has been shown that 40% of females in second or later pregnancy develop anti-HLA antibodies, 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, $35 \times 10^6$).

Previous studies have identified women with pregnancies as patients with an increased risk for HLA-alloimmunization. When leukocyte-depleted platelet transfusions, which contain, on average, $46 \times 10^6$ leukocytes, were administered, 6 of 8 patients with previous pregnancies became refractory versus none of 17 nonpresensitized patients. Previously, it had been shown that platelet transfusions containing less than $20 \times 10^6$ leukocytes induced refractoriness in 15 of 71 (21%) of females with previous pregnancies, whereas this occurred in only 16 of 264 (6%) nonpresensitized patients. In the present study, leukocyte depletion was more vigorous and we have now shown that platelet products containing less than $5 \times 10^6$ leukocytes (mean, $2 \times 10^6$) 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 present are able to induce a secondary HLA-immune response. Studies in rats and mice 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 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. In humans, Pellegrino et al 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. It was suggested in animal studies that UV-B irradiation is superior to leukocyte depletion in the prevention of the immune response.

Finally, it might be worthwhile 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. 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 depletition 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.

**ACKNOWLEDGMENT**

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REFERENCES