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 × 10⁶ per transfusion) or leukocyte-depleted random single-donor platelet transfusions. Leukocyte depletion to less than 5 × 10⁶ leukocytes per platelet transfusion (mean leukocytes, 2 × 10⁶ 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 × 10⁶ 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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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 procystatine 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 postfiltration 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 Fecht Rosenthal chamber. The detection limit of this method is $\approx 5 \times 10^{9}$/L leukocytes, which corresponds to $\approx 2 \times 10^{9}$ leukocytes per platelet transfusion.

Transfusion regimen. ABO-compatible platelets were transfused prophylactically when the platelet count dropped below 10 to 20 x $10^{7}$/L, or therapeutically for invasive procedures and in case of major hemorrhagic symptoms at platelet counts above 10 to 20 x $10^{7}$/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 (m²) 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 $-70^\circ$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 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-
of refractoriness and the time interval to develop refractori-
ness. When the patients in the filtered group were censored
from the moment of receiving a platelet product containing
more than $5 \times 10^8$ 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 transfu-
sion 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 pa-
tients, 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 sero-
conversion. The results of the analysis regarding the occur-
rence 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 anti-
bodies (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 corre-
sponds to an increase in the actuarial probability of devel-
oping anti-HLA antibodies of 14% (95% CL: $-16\%$, $+42\%$,
Cox proportional hazard model). With regard to the reactiv-
ity pattern of the anti-HLA antibodies, the median percentage
panel reactive antibodies (PRA) at the time of seroconver-
sion 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-speci-
ficities were identified. Sera of four patients in the filtered

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 376/671 (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 $>38.5\text{°C}$: $n = 2$, DIC: $n = 1$) were present; in the sera of these 3 patients, anti-HLA antibodies were demonstrable. (2) in five patients, a 1-hour posttrans-
fusion 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

Eight patients in the filtered group received a total number
of 11 platelet products containing greater than $5 \times 10^6$ leuko-
cytes. Two of these patients became refractory. The number
of leukocytes in the four transfusions administered to these
two patients varied from $5.1 \times 10^6$ to $7.0 \times 10^6$ leukocytes
with the exception of one transfusion containing $20 \times 10^6$
leukocytes.

refractoriness 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 ran-
dom multiple-donor transfusion would not have altered the
outcome of the analysis (data not shown).

In 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 num­
ber of platelet transfusions (D). Ordinate: proportion
of patients not becoming refractory (A, B) and pro­
portion of patients not forming anti-HLA antibodies
(C, D). (—), Filtered group; (—), standard group.

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of patients not becoming refractory (A, B) and pro­
portion of patients not forming anti-HLA antibodies
(C, D). (—), Filtered group; (—), standard group.
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 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. Secondly, it has been shown that 40% of females in second or further 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 depletions were 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 per se 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 deple tion 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 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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REFERENCES


