Alloimmunization to Platelets in Heavily Transfused Patients With Sickle Cell Disease

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Bone marrow transplantation (BMT) is now an option for some patients with sickle cell disease (SCD). Many SCD patients are multiply transfused with red blood cells (RBCs), and may be immunized to alloantigens other than erythrocyte antigens. Because platelet refractoriness is a significant complication during BMT, we wished to determine the prevalence of alloimmunization to platelets in transfused SCD patients. Sera collected from 47 transfused and 14 untransfused SCD patients were screened for HLA and platelet-specific antibodies. Transfusion and RBC antibody histories were reviewed. A subset of the patients were rescreened 1 year later. Eighty-five percent of patients with at least 50 RBC transfusions (22 of 26), 48% of patients with less than 50 transfusions (10 of 21), and none of 14 untransfused patients demonstrated platelet alloimmunization (P < .05). Platelet alloimmunization was more prevalent than RBC alloimmunization (20% to 30%). Half of the platelet reactivity was chloroquine-elutable. Eighteen of 22 patients (82%) on chronic RBC transfusion remained platelet-alloimmunized 11 to 22 months after initial testing. In summary, 85% of heavily transfused SCD patients are alloimmunized to HLA and/or platelet-specific antibodies. These patients may be refractory to platelet transfusion, a condition that would increase their risk during BMT. Leukodepletion in the transfusion support of SCD patients should be considered to prevent platelet alloimmunization.

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THE ONLY CURRENTLY available therapy considered a “cure” for sickle cell disease (SCD) is allogeneic bone marrow transplantation (BMT). Since the median life expectancy for SCD patients with access to medical care in the United States is 42 to 48 years, and since most patients with SCD can expect to lead functional, productive lives, physicians caring for these patients have generally judged the risks inherent to BMT too great to justify recommending transplantation for SCD except in special circumstances. However, the risk to benefit ratio of BMT in SCD has recently been reconsidered in view of the low mortality rates with transplantation for thalassemia major (<10%). Furthermore, SCD patients living in developing countries have a poorer prognosis than those in the United States if they do not have access to adequate medical care. These considerations motivated two European groups to offer BMT to selected SCD patients. They reported no mortality and minimal long-term morbidity among the first 30 transplants. The total number of SCD patients who have received marrow allografts worldwide is currently approximately 70, with approximately 30 reported from US centers. The Bone Marrow Transplantation for SCD Study Group has approved a multiinstitutional protocol to study allogeneic BMT for SCD patients aged 15 years or younger, so it is likely that an increasing number of SCD patients will undergo BMT in the near future.

Refractoriness to platelet transfusion is a difficult clinical problem that can complicate the supportive care of BMT patients. Rates of refractoriness of 40% to 70% have been reported in patients with a prolonged requirement for platelet transfusion during chemotherapy-induced marrow aplasia or marrow transplantation for malignancy. Immunologic platelet destruction mediated by alloantibodies directed against antigens on platelets is frequently the principal factor or an important contributing factor in the platelet refractory state. There is a general correlation between alloimmunization to HLA and platelet antigens and clinical platelet refractoriness, and this correlation provides the basis for blood product selection strategies in the management of refractory patients.

SCD patients who may be considered for BMT are different from other BMT candidates in several important respects that might affect their risk of developing platelet refractoriness. First, many will have received numerous red blood cell (RBC) transfusions, possibly over many years prior to transplant, as therapy for the complications of SCD. They are unlikely to have received platelet concentrates or other non-RBC blood products. Second, their transfusions will not have been given concurrently with chemotherapy or immunosuppression, as is usually the case for cancer and aplastic anemia patients. Third, the immune responses of SCD patients may differ from those of other groups of multiply transfused patients and BMT candidates for reasons inherent to their chronic illness, such as the functional asplenia that develops after the first few years of life. Finally, the problem of antigenic mismatching between blood donor and SCD recipient populations may apply to HLA and platelet-specific antigens as well as RBC antigens. Thus, it may not be valid to extrapolate from the experience with cancer, aplastic anemia, or thalassemia patients to predict the rates of platelet alloimmunization or refractoriness in SCD patients undergoing BMT.

Based on these reports and the high incidence of platelet alloimmunization in other multiply transfused populations, we hypothesized that antiplatelet antibodies would be detected frequently in transfused SCD patients. We tested this hypothesis by screening the sera of heavily transfused, moderately transfused, and untransfused SCD patients for platelet antibodies.

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PLATELET ALLOIMMUNIZATION IN SCD

MATERIALS AND METHODS

Patients were recruited for this study from the outpatient Hematology Clinic and the Therapeutic Apheresis Center of the Children's Hospital of Philadelphia, which is the site of a federal- and state-funded Comprehensive Sickle Cell Center. Patients were asked to participate if their hemoglobin genotype, as documented by hemoglobin electrophoresis and other studies, was SS, SC, or Sβ-thalassemia. Patients were excluded if they had received plasma transfusion or intravenous or intramuscular γ-globulin within the previous 6 months, if they were receiving immunosuppressive medication such as corticosteroids, if they were or had ever been pregnant, or if they had a diagnosis of human immunodeficiency virus infection or an autoimmune disease such as idiopathic thrombocytopenic purpura, systemic lupus erythematosus, or ulcerative colitis. If the patient was febrile or suspected of having an infection, collection of blood for the study was deferred until the patient was well. A mild painful episode alone was not an exclusion criterion.

Patients or their families who gave written informed consent to participate as approved by the hospital Institutional Review Board were divided into three groups according to transfusion history. Group 1 subjects were heavily transfused children and young adults currently on a chronic transfusion protocol who had received at least 50 RBC transfusions in their lifetime and who had received RBC transfusions every 2 to 5 weeks for at least 6 months prior to enrollment. Serum was collected from these patients just before a scheduled transfusion. Group 2 contained moderately transfused patients with one to 49 RBC transfusions either on a sporadic basis or as part of a recently initiated regular transfusion program. Serum was collected from these patients at routine clinic visits or just before a scheduled transfusion. Group 3 subjects were SCD patients who had never been transfused, as determined by history and review of blood bank records. Serum was collected at routine clinic visits.

Whole-blood samples were drawn into tubes without anticoagulant at the time of venipuncture for clinical testing. Blood was separated by centrifugation, and the serum was transferred to a fresh tube, labeled, and stored at −20°C until use.

Assay for platelet alloantibodies. The Capture-P Ready Screen (Immucor Inc, Norcross, GA) solid-phase RBC adherence (SPRCA) assay for detection of IgG antibodies on platelets was used to screen for HLA or platelet-specific antibodies. The kit contains strip wells configured as 96-well plates to which platelets from 12 different donors have been fixed. The panel of platelets is selected to include common HLA types plus the platelet-specific antigens, HPA-la, HPA-1a, HPA-1b, HPA-3a, HPA-3b, HPA-4a, HPA-5a, and HPA-5b, and is designed for screening for platelet alloimmunization but not for definitive identification of antibody specificity.

The kit was used according to the manufacturer's procedure, using an automated multichannel dispenser and centrifuge supplied by the manufacturer. The results were read visually on a transilluminator and recorded by a single individual (M.B.L.), and were reviewed by a second observer (D.F.F.). A positive reaction was defined as a film of indicator RBCs in the well or a pellet of indicator RBCs with a diffuse edge after centrifugation. A negative reaction was defined as a pellet of indicator cells with a tight, sharp edge after centrifugation. Strong positive, weak positive, and negative control sera supplied with the kit were assayed with each strip. In addition, positive control sera from patients known to have anti-HLA or anti-platelet antibodies by standard cytotoxicity assays were obtained from the reference laboratory of the American Red Cross Blood Services–Penn Jersey Region and shown to be positive in this assay.

Chloroquine elution of HLA class I antigens from the immobilized platelets was used as a method to distinguish serum reactivity to HLA antigens versus platelet-specific antigens. Chloroquine elutes HLA antigens from the platelet membrane, reducing or eliminating reactivity to HLA, but does not affect platelet-specific antigens.11,12

Using the procedure recommended by the manufacturer for the SPRCA assay, 2 drops of 16% chloroquine diphosphate (Gamma Biologicals, Houston, TX) and 1 drop of saline solution were added to each well, incubated for 30 minutes at 37°C, and removed by washing with phosphate-buffered saline. The serum was then tested as described earlier, using both chloroquine-pretreated wells and untreated wells in parallel to control for variables such as specimen age, freeze-thaw cycles, and potential run-to-run variation in centrifugation and reading of the results. The number of positive wells in chloroquine-pretreated assays was subtracted from the number of positive wells in the simultaneous untreated assays to estimate the fraction of platelet reactivity solely due to HLA specificities.

Study design and statistical analysis. A patient was considered to be alloimmunized to platelets if at least two of 12 test wells were positive.

The study was designed to compare 40 chronically transfused SCD patients (group 1) with 30 untransfused SCD patient controls (group 3) in order to have 80% power to demonstrate with 95% confidence (P < .05) a rate of alloimmunization in group 1 of 50% with a confidence range of ±15%, and in group 3 of less than 10%. Smaller numbers of patients were actually needed, because no alloimmunization was detected in the first 14 patients in group 3 and because the actual rate of alloimmunization in group 1 was 85% rather than 50%. Patients in group 2 were studied initially to develop a qualitative description of the correlation between number of transfusions and degree of alloimmunization as measured by the number of wells positive (of 12) in the SPRCA assay. Statistical analysis of the difference in prevalence of platelet alloimmunization between the three groups was performed with a two-tailed Fisher's exact test, and analysis of the correlation between number of positive platelet wells and number of transfusions received was performed with the Spearman test of correlation. The effect of chloroquine on the reduction in the number of positive wells was analyzed with the Wilcoxon matched-pair signed-rank test. Analysis of the association between alloimmunization to platelets and to RBCs was performed with Fisher's exact test.

RESULTS

Sample. A total of 61 samples were obtained from 30 male and 31 female patients with SCD. There were 26 patients with at least 50 transfusions (group 1), 21 patients with one to 49 RBC transfusions (group 2), and 14 untransfused SCD patients (group 3). The groups were dissimilar with regard to age distribution, with mean ages of 18.3 ± 5, 8.7 ± 6, and 4.8 ± 4 years in groups 1, 2, and 3, respectively (Fig 1), reflecting the age of onset of complications requiring transfusion (principally stroke) and the duration of chronic transfusion therapy required to accumulate at least 50 transfusions. Figure 1 also shows the distribution of the number of transfusions on the abscissa. For 22 patients in group 1, these figures are underestimate of the number of transfusions received, because transfusion records prior to 1990 were not available in some cases; however, for all patients in group 1, at least 50 transfusions were documented. Complete records were available for all patients in groups 2 and 3.

Prevalence of platelet alloimmunization. The prevalence of alloimmunization to platelets (± two positive wells out of 12 in the SPRCA assay) in the three groups is shown in Table 1. None of the patient sera in untransfused group 3 had even one well positive, whereas 10 of 21 (48%) in group

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2 and 22 of 26 (85%) in group 1 had two or more wells positive ($P < .02$).

For the 47 transfused patients in groups 1 and 2 (Fig 2A), there was a significant positive correlation between the number of positive SPRCA wells and the number of transfusions ($r = .49$, $P < .001$). The correlation of positive wells with the number of transfusions was not significant when the analysis was restricted to group 1 alone ($n = 26$, $r = .23$, $P = .23$) or group 2 alone ($n = 21$, $r = .32$, $P = .15$). The distribution of positive wells in the SPRCA assay for groups 1 and 2 is shown in Fig 2B and C. In group 1, 15 of 26 (57%) had at least 9 of 12 wells positive, whereas half of group 2 had no wells positive and only 4 of 21 (19%) had at least 9 wells positive.

Chloroquine pretreatment. Thirty-eight samples from groups 1 and 2 were tested for platelet antibodies with both untreated and chloroquine-pretreated wells, to distinguish between sera with specificities for HLA antigens only versus sera that might contain antibody to platelet-specific antigens (Fig 3). Six samples that were entirely negative and two with one well positive in the untreated assay were included as controls. In 30 samples that had at least two wells positive in the untreated test (of 32 total initially positive samples, the remaining two having been exhausted), chloroquine pretreatment reduced the number of positive wells in 27 samples, showed no change in two samples, and produced an increase in one sample (from 9 to 10 wells). These 30 sera yielded a total of 256 positive untreated wells, of which 122 remained positive with chloroquine pretreatment. In summary, 27 of 30 (90%) transfused SCD patients with platelet alloimmunization had evidence of anti-HLA specificity as determined by chloroquine elution. HLA specificities accounted for at least 52% of this platelet alloantibody activity (134 of 256 chloroquine-elutable wells); the remaining 48% (122 of 256 nonelutable) may be additional HLA specificities or platelet-specific antigen specificities.

Persistence of alloimmunization. Follow-up serum samples were obtained from 22 patients of groups 1 and 2 who were initially SPRCA-reactive, 11 to 22 months after the initial specimen. All of the patients had remained on a chronic transfusion program with simple RBC transfusions or automated partial RBC exchange every 3 to 4 weeks during the follow-up interval. Eighteen of 22 (82%) remained alloimmunized to platelets and 4 of 22 lost SPRCA reactivity. Of these 4, 2 had shown 10 wells positive in the initial sample (Fig 4). Thus, platelet alloimmunization persists after 1 year in 82% of this heavily transfused SCD population.

Follow-up results for five additional patients from groups 1 and 2 who were not initially SPRCA-reactive and who received regular transfusions during the follow-up interval are also shown in Fig 4. Three became reactive and two remained nonreactive despite ongoing transfusion exposures.

**RBC versus platelet alloimmunization.** Transfusion records for 45 of 47 patients in groups 1 and 2 were reviewed for evidence of alloimmunization to RBC antigens (no RBC antibody data were available for two patients). We included any antibody specificity that could be considered humoral response to an antigen on RBCs, including clinically significant RBC alloantibodies in the Rh, Duffy, Kidd, Kell, and MNS systems, as well as less significant specificities such as Lewis, HLA antibodies, and Bg (presumed HLA antibodies detected in RBC agglutination tests). Cold agglutinins with autospecificities such as anti-I were not counted as alloimmune responses. Using this inclusive definition, the prevalence of any RBC antibody was 40% (18 of 45), less than
the prevalence of platelet alloimmunization of 69% (31 of 45) in the same group 1 and group 2 patients (Table 2). If a more restricted definition of RBC alloimmunization were used, omitting presumed HLA specificities, two patients would be reclassified as lacking RBC antibodies, for a prevalence of RBC antibodies of 36% (16 of 45). Table 2 shows that there was no statistically significant association between RBC alloimmunization and platelet alloimmunization, as defined either by at least two wells positive (any platelet alloimmunization) or by nine wells positive (broad platelet alloimmunization). The mean number (± standard deviation) of positive platelet wells for patients with RBC alloimmunization was 7.2 ± 4.7, and for patients without RBC alloimmunization, 5.0 ± 4.2, but this difference was not statistically significant by Student’s t test (n = 18, t = 1.6, P = .12).

DISCUSSION

Refractoriness to platelet transfusion is a significant cause of morbidity and mortality following BMT. Alloimmunization to HLA antigens and to platelet-specific antigens, induced by transfusion of cellular blood products, is one of several factors that can contribute to the platelet refractory state. Because episodic or chronic RBC transfusion therapy is used in the management of serious complications of SCD such as stroke, acute chest syndrome, priapism, and intractable pain, many of the SCD patients who would currently be considered potential candidates for BMT have been heavily transfused.

There are no published studies that have directly examined platelet alloimmunization in transfused SCD patients. One study reported that 73% of children with SCD who had received transfusions had antilymphocyte antibodies. Such antilymphocyte antibodies probably included anti-HLA antibodies and might be detected as antiplatelet antibodies by current techniques. Another study from Greece reported platelet antibodies using two different methods in 51.9% of heavily transfused thalassemia patients. Finally, a study of transfused renal transplant recipients (non-SCD) showed a
correlation between the presence of RBC antibodies and HLA antibodies.\textsuperscript{15}

In this study, we observed that both heavily transfused (\(\geq 50\) transfusions) and moderately transfused (one to 49 transfusions) SCD patients have a high prevalence of platelet alloimmunization — 85% and 48%, respectively — compared with untransfused SCD patients. The 52% reduction in reactivity with chloroquine pretreatment suggests that at least half of the antibodies detected are directed against HLA antigens. Platelet-specific antigens may account for a portion of the remaining 48%. This high prevalence of platelet alloimmunization remains detectable in the majority (82%) of patients during the time they remain on RBC transfusion programs.

These findings suggest that heavily transfused SCD patients might have significant platelet refractoriness during BMT. Furthermore, refractoriness might develop earlier in the course of transplant for SCD than for malignant disease, since most SCD patients would already be heavily alloimmunized to platelets at the time of conditioning. The SPRCA technique we used has been shown to detect antiplatelet antibodies in alloimmunized patients\textsuperscript{16} and in idiopathic thrombocytopenic purpura patients.\textsuperscript{17} The method has also been used for platelet crossmatching in refractory patients and provides results that correlate with improved posttransfusion platelet increments.\textsuperscript{16,18,19} Thus, it is likely that the SPRCA reactivity demonstrated in transfused SCD patients will be predictive of an increased risk of platelet refractoriness, and perhaps an increased risk of significant hemorrhage, if these patients undergo BMT.

The test of this hypothesis would require careful analysis of the response to platelet transfusions and the rate of thrombocytopenic bleeding complications in a series of SCD patients undergoing transplant. The only published report that addresses these issues\textsuperscript{20} describes two SCD patients with thrombocytopenia and intracranial hemorrhage after BMT. These two, as well as five others who were not thrombocytopenic and who developed seizures after BMT, had a history of cerebrovascular accident (CVA) and had been on chronic transfusion therapy before transplant. The investigators concluded that prior CVA was a significant risk factor for neuro-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{Chloroquine pretreatment to elute HLA antigens from the SPRCA assay reduces serum reactivity in transfused SCD patients. Thirty-eight serum samples from groups 1 and 2 were tested in SPRCA wells untreated or pretreated with chloroquine, to estimate the proportion of platelet alloimmunization attributable to HLA specificities v. platelet-specific antigens. The number of positive wells in untreated and treated tests (always performed in parallel) is plotted as a line for each serum. Chloroquine pretreatment results in a significant reduction in the number of positive wells (\(P < .001\), Wilcoxon matched-pairs signed-ranks test), suggesting that HLA specificities contribute significantly to the observed platelet alloimmunization in transfused SCD patients.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Persistence of platelet alloimmunization over \(1\) year of ongoing chronic transfusion therapy. The number of positive SPRCA wells at initial and follow-up testing is plotted for 27 patients in groups 1 and 2, all of whom received ongoing chronic transfusion therapy during the follow-up interval. To the right of the vertical dotted line are 22 patients who were alloimmunized on initial testing (\(\geq 2\) wells positive), of whom 18 remained alloimmunized and 4 lost detectable platelet antibody at follow-up. To the left of the vertical dotted line are 5 patients who were not alloimmunized initially and were tested after 1 year of ongoing transfusion therapy; of these, 3 became alloimmunized and 2 did not.}
\end{figure}

\begin{table}[h]
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\caption{Association of Platelet and RBC Alloantibodies in Transfused SCD Patients}
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Parameter & Positive & Negative & Total & \(P\) \\
\hline
Platelet antibody-positive & 13 & 18 & 31 (69\%) & NS \\
Platelet antibody-negative & 5 & 9 & 14 & \\
\(\geq 9\) wells reactive & 10 & 9 & 19 & NS \\
<9 wells reactive & 8 & 18 & 26 & \\
Total & 18 (40\%) & 27 & 45* & \\
\hline
\end{tabular}
\begin{flushleft}
\textsuperscript{*}No significant correlation (NS) was detected by Fisher's exact test or by the chi-square test.
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\end{table}
logic sequelae in transplanted SCD patients, and believed that platelet refractoriness may have been contributing cause of intracranial hemorrhage in two cases.

Alloimmunization to RBC antigens has been shown to occur in 18% to 36% of transfused SCD patients.\textsuperscript{11,21,22} Using an inclusive definition, we found that 40% of transfused SCD patients in this study had RBC alloimmunization. This prevalence of alloimmunization to RBC antigens is higher than observed in studies of other chronically transfused populations such as thalassemics.\textsuperscript{23,24} One explanation for the higher prevalence of RBC alloimmunization in SCD is the greater likelihood of antigenic mismatching between the blood donor population, which in the United States is predominantly white, and the recipient (patient) population.\textsuperscript{18} (SCD patients are predominantly African-American, whereas thalassemia patients are predominantly white). In addition, in both populations, individual patients appear to be predisposed to the formation of numerous RBC antibodies (responders), whereas others with the same degree of allelic exposure form few or none (nonresponders). In this study, we found a high prevalence of alloimmunization to both RBC and platelet antigens in transfused SCD patients, but no significant association between RBC and platelet alloimmunization. This suggests that responders to RBC antigens are not more likely to respond to the antigenic determinants responsible for platelet alloimmunization.

The persistence of platelet reactivity in 82% of SCD patients who continued to receive regular RBC transfusions distinguishes them from other chronically transfused populations. HLA antibodies become undetectable in 30% to 66% of initially alloimmunized cancer patients, even if they continue to receive transfusions.\textsuperscript{26,27} This difference in the persistence of platelet alloimmunization may be related to differences in underlying disease, in immunologic function, or in the lack of immunosuppressive therapy in SCD patients compared with cancer or BMT patients. The type of ongoing transfusion therapy given to SCD patients versus cancer or BMT patients is also different, since SCD patients receive almost exclusively RBC products.

Finally, these findings raise the issue of leukodepletion of RBC products for transfusion to SCD patients. Current theories about the mechanism of transfusion-induced alloimmunization to platelets suggest that residual white blood cells in cellular blood products play a key role in presenting foreign HLA and platelet antigens to the recipient’s T and B cells, stimulating the antibody response to foreign HLA substances. Furthermore, there is evidence that leukodepletion by filtration of blood products given to cancer patients or BMT recipients may reduce or delay the onset of HLA alloimmunization, also reducing or delaying the onset of the refractory state,\textsuperscript{18,29-32} although the efficacy of leukodepletion in reducing platelet alloimmunization in these patients is still controversial.\textsuperscript{33}

As already discussed, SCD patients differ in several ways from the patients in whom this benefit of blood product leukodepletion has been suggested. The high prevalence and persistence of platelet alloimmunization demonstrated here in heavily transfused SCD patients suggests that as a group they may be more immunocompetent than heavily transfused cancer or BMT patients. For this reason, leukodepletion of blood products might be less efficacious in SCD patients than in cancer and BMT patients for prevention of platelet alloimmunization. However, for the same reason, prevention of platelet alloimmunization may be especially important for SCD patients who ultimately have BMT. Thus, it may be appropriate to consider extending the indication for leukoreduction of blood products for the prevention of platelet alloimmunization to include some SCD patients as potential BMT recipients.

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