Therapeutic Effectiveness of Frozen Platelet Concentrates for Transfusion

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Six patients received platelet concentrate transfusions from their HLA-identical siblings. Platelet concentrates were administered either fresh, or after being frozen in 10% dimethylsulfoxide, at a slow controlled rate (1°C/min) or rapidly (~ 8°C/min) in the vapor-phase of a liquid nitrogen refrigerator. The median freeze-thaw loss was 13.5%. The mean 1-hr and 20-hr corrected increments in platelet count were calculated for fresh platelet concentrates transfused before and after transfusion with controlled-rate frozen and vapor-phase frozen platelet concentrates. There was no significant difference among the first and second transfusion of fresh platelet concentrates, nor was the difference observed between fresh and controlled-rate frozen platelet concentrates significant.

Platelet Concentrates

Platelet concentrates from the normal sibling were prepared by the standard manual four-unit plateletpheresis method employing ACD anticoagulant followed by a final centrifugation to reduce leukocyte contamination. The final product had a median concentration of 0.71 x 10^11 platelets/U (range 0.55 to 1.04 x 10^11). Platelet counts were performed using a Coulter Counter (ZBI Model, Coulter Electronics, Hialeah, Fla.). If the platelet concentrate was to be cryopreserved, the following additional processing was used. The four-unit platelet concentrate was centrifuged at 3000 g for 6 min (Sorvall RC-3, room temperature), and the platelet-poor plasma was expressed into a 300 ml transfer bag (Fenwal 4R2014, Morton Grove, Ill.), resuspending the platelets in a final volume of 50 ml. The platelet-poor plasma was then frozen (~ 80°C) for use at the time of thawing.

Platelet Cryopreservation

Dimethylsulfoxide (DMSO) was used as the cryoprotectant. The cryopreservative solution (20% DMSO, 20% donor plasma, and 60% Hanks' balanced salt solution) was prepared and kept in an ice-bath. Fifty milliliters of the chilled cryopreservative solution and 50 ml of the platelet concentrate were mixed and placed in two polyolefin freezing bags (Union Carbide, Style #2030-2, Chicago, Ill.) and the bags were heat-sealed. This process resulted in a final concentration of 10% DMSO with one-half the donor platelets in each bag. The difference between fresh and vapor-phase frozen platelet concentrates, and between controlled-rate frozen and vapor-phase frozen platelet concentrates were highly significant (p < 0.01). In vitro tests of aggregation using ristocetin and platelet ultrastructural studies paralleled the transfusion experience. Our results indicate that HLA-identical platelet concentrates can be successfully frozen and thawed for transfusion if a slow, controlled rate of freezing is employed. The use of HLA-identical frozen platelet concentrates may be important in emergency situations for the refractory patient and potentially for the establishment of a platelet concentrate bank.

MATERIALS AND METHODS

Patients

Six patients (2 aplastic anemia, 2 acute nonlymphoblastic leukemia, 1 acute lymphoblastic leukemia, and 1 multiple myeloma), who had an HLA-identical sibling and were refractory to random-donor platelet concentrates, were studied. The HLA typing was performed by a modification of the microdroplet lymphocyte cytotoxicity test described by Mital. Patients were without evidence of fever, sepsis, overt bleeding manifestations, or splenomegaly, and were not taking medications associated with decreased platelet survival. Chemotherapy was not being administered during this period. These studies were reviewed by the Institutional Review Board for Human Investigation and patients and donors gave their informed consent.
two bags were immediately placed between two aluminum cover plates and fastened with exterior clamps. Final bag thickness measured 1–2 mm.

Freezing was carried out in two ways. Vapor-phase freezing involved placing the freezing bag vertically in a liquid nitrogen refrigerator just above the level of liquid nitrogen (−170°C). After 1 hr in the vapor phase, the frozen platelets were removed and placed in the liquid phase of the liquid nitrogen refrigerator. Controlled-rate freezing was performed using a biological freezer (Union Carbide, Linde, Chicago, Ill.). The freezing rate was monitored by a copper thermocouple probe, recording on a temperature recorder (Esterline Angus, Indianapolis, Ind.). Initial cooling rate was 1°C/min until the heat of fusion (the temperature at which liquid state to frozen state change of phase occurs) was reached, at which time a boost, a second rate of cooling (1–2°C/min) was used until −100°C was reached, at which time the bags were transferred to the liquid phase in the liquid nitrogen refrigerator for storage. Characteristic freezing curves are illustrated in Fig. 1. Platelet concentrates were stored from several days to weeks before thawing. The frozen platelet concentrates contained a median concentration/U of 0.97 × 10^10 platelets (range 0.70–2.42 × 10^10) for controlled-rate freezing and 0.92 × 10^10 platelets (range 0.70–1.71 × 10^10) for vapor-phase freezing.

Thawing

Frozen platelet concentrates were submerged in a 37°C water bath until thawing was complete. Platelet concentrate samples were obtained for counts. Using donor platelet-poor plasma, another sample was diluted to a concentration of 250,000 platelets/μl for vitro testing of pH and aggregation, and for wet mount preparations and electron microscopy. The remaining platelet concentrate was added to 200 ml of donor plasma for transfusion to the patient. The freeze-thaw loss was calculated based on the platelet counts obtained at the time of freezing and thawing according to the formula:

\[
\% \text{ Loss} = \frac{\text{PC}_f - \text{PC}_t}{\text{PC}_t} \times 100
\]

where \(\text{PC}_f\) was the platelet count at the time of freezing and \(\text{PC}_t\) was the platelet count at the time of thawing. The freeze-thaw loss was similar for controlled-rate freezing and for vapor-phase freezing: median loss 13.5% (range 0%–22%).

Platelet Transfusion

Platelets were transfused through a standard component administration set (Fenwal 4C 2100, Morton Grove, Ill.). Fresh platelet concentrates were administered within 6 hr of collection and frozen platelet concentrates within 30 min of thawing. Platelet counts on peripheral blood were performed on patient blood samples obtained immediately before, 1 hr, and 20 hr after transfusion. The fresh platelet concentrates were transfused first, followed on subsequent days by the vapor-phase frozen and controlled-rate frozen platelets. Transfusion with fresh platelet concentrates was repeated a second time to demonstrate that satisfactory post-transfusion platelet increments were still achieved, thus bracketing the frozen platelet transfusion experience.

Platelet Studies

In vitro studies included platelet aggregation, pH, and examination of wet mount preparations. Electron microscopy of platelet concentrates was also performed. Fresh platelets and thawed, frozen platelets were studied in tests of aggregation (Bio-Data Platelet Aggregation Profiler Model PAP-2A, Willow Grove, Penn.). One-half of a milliliter of platelet concentrate was diluted with donor plasma at room temperature to give a final concentration of platelet-rich plasma containing approximately 250,000 platelets/μl, and changes in optical density on a 0.45 ml sample (relative to 0.45 ml of platelet-poor plasma) were recorded and a percent aggregation was calculated. Ristocetin (Cutter Laboratories, Berkeley, Calif.) was the aggregating agent. Preliminary experiments using ADP (adenosine diphosphate), epinephrine, thrombin, and collagen as aggregating agents showed poor aggregation of all platelet concentrates including fresh, probably because of the high citrate concentration. Therefore, ristocetin was selected for study. The pH of the adjusted platelet-rich plasma was measured using a standard pH meter. The pH of all concentrates was between 6.8 and 7.2. Wet mount preparations at 37°C were performed using one drop of adjusted platelet-rich plasma on a glass slide with a silicone-coated pipette and observed under a phase microscope noting the percentage of discs, spheres, dendrites, and balloons.

For electron microscopy, 3-ml samples of fresh and thawed, frozen platelet concentrate were transferred to 10 ml aliquots of fixative containing 2.5% glutaraldehyde buffered with 0.05 M cacodylate buffer and 4% sucrose. These samples were additionally fixed in 1% osmium tetroxide in 0.1 M S-Collidine buffer for 1 hr, and en bloc stained in 1% aqueous uranyl acetate for 30 min. Then the material was dehydrated in acetone and embedded in Spur; later sections were cut with diamond knives on a Sorwall Porter-Blum MT 2-B ultramicrotome, stained with 1% uranyl acetate and lead citrate, and examined with a Phillips 201 electron microscope.

In vivo tests included bleeding times and measurement of the platelet count increment after transfusion. Bleeding times were performed using the modified Duke method with a sterile #11 surgical blade on the tip of the third finger immediately before, 1 hr, and 20 hr after transfusion. This method has been found to be quite simple and reproducible. Corrected increments were calculated, based on the following formula:

\[
\text{C.I.} = \frac{\text{observed increment} \times \text{BSA}}{\text{Number of platelets} \times 10^{11}}
\]

in which the observed increment was the difference between the pre- and post-transfusion platelet counts at a given time interval (1 hr

Fig. 1. Characteristic freezing curves of vapor phase frozen and controlled-rate frozen platelet concentrates. The solid line represents the curve obtained for controlled-rate freezing; the dashed line, the curve for vapor-phase freezing.
and 20 hr), and BSA was the estimated body surface area in square meters (sq m).

Statistics
Student's t test was employed.

RESULTS
Twenty-four HLA-identical platelet concentrate transfusions were given to 6 stable thrombocytopenic patients. In all cases, the patients had compatible transfusion responses with fresh platelet concentrates. Since the frozen platelet concentrate transfusions were bracketed by the transfusion of fresh platelet concentrates, comparisons of freezing techniques to each other and to fresh platelet concentrates were possible. Figure 2 depicts the transfusion responses measured at 20 hr after transfusion for the 24 transfusions in the 6 patients. The values obtained for the two fresh platelet concentrate transfusions are shown separately. Transfusions with fresh platelet concentrates initially resulted in a mean corrected platelet increment (platelets/μl per sq m/10^11 platelets transfused) of 14,600 (±1800 SEM) 20 hr after transfusion. The second transfusion with fresh platelet concentrates, given after frozen platelet concentrates, resulted in a mean corrected increment of 14,800 (±1500). Controlled-rate frozen platelet concentrate transfusions achieved a mean 20-hr corrected increment of 12,500 (±1600); the difference between fresh platelet concentrates and controlled-rate frozen platelet concentrates was not statistically significant. The mean corrected increment obtained with the platelet concentrates frozen in the vapor phase was 2600 (±1000) 20 hr after transfusion. Comparisons of fresh platelet concentrates and vapor-phase frozen platelet concentrates, and of controlled-rate frozen platelet concentrates and vapor-phase frozen platelet concentrates were highly significant (p < 0.005). Higher post-transfusion increments were observed when measured at 1 hr, and the pattern of the results was similar. For first transfusion of fresh platelet concentrates the mean corrected increment at 1 hr was 22,200 (±2300); the second transfusion of fresh platelet concentrates was 25,200 (±2500), p = NS. Controlled-rate frozen concentrate transfusions resulted in a mean 1-hr corrected increment of 22,900 (±5100); vapor-phase frozen concentrates only 7600 (±900). Again, the difference between fresh platelet concentrates and controlled-rate frozen platelet concentrates was not statistically significant. Increments with fresh platelet concentrates compared to vapor-phase frozen platelets and increments with controlled-rate frozen platelet concentrates compared to vapor-phase frozen concentrates were significant (p < 0.01).

In one patient, the bleeding time was measured immediately before and 20 hr after transfusion of fresh, controlled-rate frozen, and vapor-phase frozen platelet concentrates. Correction of the prolonged bleeding time (>15 min) to <6 min was observed when fresh and controlled-rate frozen platelet concentrates were transfused. In contrast, upon transfusion of vapor-phase frozen platelet concentrate, only a modest decrease was observed (>15 min to 10 min). The changes in bleeding times were directly related to increments in platelet count after transfusion. Bleeding times were not performed in the other patients because of severe neutropenia and the potential risk of infection.

In vitro platelet studies of wet mount morphology revealed the fresh platelet concentrates to be nearly all disc forms and were superior in appearance to either of the frozen platelet concentrates (Table 1). Platelets frozen at a controlled-rate contained discs but dendrite and sphere forms predominated. Balloon forms as well as greater numbers of dendrites were noted in platelet concentrates frozen in vapor-phase.

Table 1. Wet Mount Morphology of Platelet Concentrates

<table>
<thead>
<tr>
<th>Platelet Concentrate</th>
<th>Disc</th>
<th>Sphere</th>
<th>Dendrite</th>
<th>Balloon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh (12)</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(60-95) (15-30) (0-10) (0-0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Controlled-rate frozen (6)</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>(0-20) (40-80) (20-60) (0-0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vapor phase frozen (6)</td>
<td>0</td>
<td>25</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>(0-10) (0-50) (20-60) (0-20)</td>
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*Number of observations in parentheses.
†Median, range in parentheses.

Fig. 2. Comparison of transfusion response using fresh and frozen platelet concentrates. The shaded bars represent the first and second transfusion response with fresh platelet concentrates (1st FRESH and 2nd FRESH). The open bars represent the transfusion response with controlled-rate frozen (CRF) and vapor-phase frozen (VPF) platelet concentrates. The height of the bars denote the mean increment; the symbol (±) denotes the standard error of the mean. Corrected increments were calculated on the observed 20-hr post-transfusion platelet increments.
Electron microscopy of fresh concentrates showed mainly disc forms. The ultrastructural appearance of platelets frozen in vapor-phase (Fig. 3) showed an admixture of discoid, sphere, and occasional balloon forms. The balloon forms revealed disrupted plasma membrane, and loss of the matrix, microtubules, and canalicular systems. Dilated canalicular systems were noted in the sphere forms. Platelets frozen at a controlled-rate showed many sphere forms and occasional discoid forms, but no balloon forms were observed (Fig. 4). The sphere forms showed prominently dilated canalicular systems but no loss of the cellular organelles was observed.

The results of platelet aggregation using ristocetin are represented in Table 2. Fresh platelet concentrates had superior aggregation compared to both controlled-rate frozen and vapor-phase frozen platelet concentrates ($p < 0.001$). The controlled-rate frozen platelets aggregated significantly better than vapor-phase frozen platelets ($p < 0.001$). This pattern of aggregation correlated with the in vivo transfusion response.

**DISCUSSION**

Platelet concentrate transfusions have gained wide acceptance in preventing thrombocytopenic hemorrhage.\textsuperscript{1-10,14-17} The use of cryopreserved platelet concentrates from compatible donors seems warranted because of the difficulty in providing compatible platelet concentrate transfusions for the highly alloimmunized patient and because of the limited viability of platelet concentrates stored in the liquid phase. The transfusion experience with previously frozen platelet concentrates has been quite variable.\textsuperscript{18-23} Schiffer and coworkers were able, in several studies, to freeze and store for long periods of time autologous platelet concentrates obtained from patients with acute leukemia in remission.\textsuperscript{18-20,29} During periods of chemotherapy-induced thrombocytopenia these autologous

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Electron microscopy of platelets frozen in vapor-phase showing discoid, sphere, and balloon forms. $\times$ 16,450.}
\end{figure}
platelets were thawed and reinfused. These authors demonstrated that the recovery and transfusion response was unrelated to the method of cryopreservation. They suggested that controlled-rate freezing was not superior to rapid, vapor-phase freezing.

Our study supports the use of cryopreserved platelet concentrates, but points out the importance in the method of freezing. Controlled-rate freezing of the platelet concentrates resulted in better post-transfusion recovery (approaching that obtained with fresh platelet concentrates) compared to platelet concentrates frozen rapidly in the vapor phase of liquid nitrogen. Our experience differs from Schiffer's et al. more recent study in that we did not use autologous platelet concentrates, but rather, we used the platelets from an HLA-identical sibling. By using the HLA-identical sibling, we avoided the problem of abnormalities in platelets from patients with acute leukemia. This problem, however, does not sufficiently explain the differences in our studies. Schiffer's group washed the platelet concentrates after thawing to remove excess DMSO. Because of problems with platelet clumping we did not wash the thawed platelets. Rather, we rapidly diluted the thawed concentrate with donor plasma to reduce the final concentration of DMSO to <2%. The initial concentration of DMSO (10%) may be questioned, since most other studies employed 5% concentration. Schiffer has shown that in concentrations up to 10% minimal changes in platelet aggregation occur.

Table 2. Platelet Aggregation with Ristocetin

<table>
<thead>
<tr>
<th>Platelet Concentrate</th>
<th>% Aggregation‡</th>
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<tr>
<td>Fresh (12)</td>
<td>75.7 ± 3.6</td>
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<tr>
<td>Controlled-rate frozen (6)</td>
<td>53.5 ± 1.3</td>
</tr>
<tr>
<td>Vapor phase frozen (6)</td>
<td>33.3 ± 5.0</td>
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</table>

*Number of observations in parentheses.  ‡Mean ± SE.
let function are observed. Murphy et al. reported that the t½ of platelets exposed to increasing concentrations of DMSO, was essentially the same if 5% and 10% DMSO was used. A 10% DMSO freezing solution was selected since it is the same concentration we have employed in our autologous bone marrow storage-transplant program. There have not been any adverse effects observed in this setting. In dogs, DMSO has been found to cause lenticular opacities at very high concentrations, but this has not been the case in humans. Also, our frozen platelet concentrates contained more platelets/bag than did Schiffier's. The role of the concentration of platelets actually frozen was not examined in either study. Finally, the temperature in the vapor phase of liquid nitrogen in the refrigerator may be important, i.e., with a lower temperature, more rapid freezing and potentially greater platelet damage may occur. At rates >5°C/min, Murphy and coworkers demonstrated progressively lower yields (increased platelet damage). The temperature of -120°C in Schiffier et al. yielded ~10°C/min, while -170°C in our study yielded a comparable ~8°C/min. Our data support studies of Murphy et al. and Valeri et al. that demonstrated improved in vivo platelet recovery with slower, controlled-rate freezing. A definite advantage in the present study is the transfusion of fresh platelet concentrates before and after the transfusion experience using frozen platelet concentrates. Using this approach, the same donor-recipient pairs were examined for each transfusion, thus eliminating much patient variability. The incremental transfusion data obtained reflected changes due to the transfused product rather than changes in transfusion status.

The in vitro studies paralleled the in vivo experience. Wet mount morphology, as well as a more objective assessment, platelet ultrastructural analysis using electron microscopy, indicated that freezing damage was minimized with a gradual rather than rapid cooling process. Since the fresh platelet concentrates aggregated normally with ristocetin, the von Willebrand's factor was present in adequate concentration. Among the changes observed with electron microscopy were disruptions in the plasma membrane. This finding would account for the altered response to ristocetin. Modifications, therefore, in the freezing technique apparently conferred increased protection to the platelet membrane. This observation corroborates the findings of Meryman et al. who noted the plasma membrane to be the primary site of freezing injury. Ristocetin aggregation and favorable platelet morphology do not assure adequate in vivo function of platelets. Although good correlation was observed in the one patient in whom bleeding times were performed, validation of the proposed methods of freezing will require performance of bleeding times on additional patients.

Although the use of a programmable freezing chamber to control the rate of freezing at 1°C/min is more expensive, more time-consuming, and more cumbersome, compared to simply placing the platelet concentrates in a liquid nitrogen refrigerator, we conclude that the slow, controlled rate of freezing is important in maintaining functional platelets for transfusion after thawing. The use of HLA-identical frozen platelet concentrates may be important in emergency situations. More widespread application may include the storage of platelets from known HLA-typed donors for use in highly alloimmunized recipients.

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