Selective Isolation of Young Erythrocytes for Transfusion Support of Thalassemia Major Patients

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Transfusion-induced hemochromatosis remains a major therapeutic complication in the management of thalassemia major patients. Using available blood cell component separators, a system has been devised to selectively harvest young red cells for transfusion support of these subjects. Red cell units isolated by this method have an average estimated mean cell age of 30 days, compared to 60 days for unfractionated blood, and contain 80% of the hemoglobin content of standard red cell units. Radiochromium half-life for young cells measured in 7 asplenic thalassemia major patients averaged 47.4 days compared to 29.5 days for routine frozen red cells. The enhanced survival is not due to reticulocyte enrichment alone, but represents a true cohort of younger red cells. Although costly, this modality could theoretically halve the transfusion requirement in transfusion-dependent patients. When combined with modern iron chelator regimens, it may be possible to achieve consistently negative iron balance prior to the onset of hemochromatosis.

Red cell transfusion continues to be a mainstay of therapy in the treatment of the chronic anemias. For many patients the benefit of maintaining a sufficient hemoglobin level between transfusions to permit normal growth and function must be weighed against the risk of transfusional hemochromatosis. This therapeutic dilemma usually leads to the conservative use of red cell transfusion, with the result that any delay in the onset of iron overload is achieved at the expense of maintaining suboptimal hemoglobin concentrations.

Maintenance of a hemoglobin concentration at less than 10 g/dl is particularly deleterious to the thalassemia major patient. Because of the excessive morbidity associated with undertransfusion in this disease, specially designed “hypertransfusion regimens” were developed. This approach has resulted in prolonged survival and improved clinical status for these individuals.

In spite of these gains, transfusion-induced hemochromatosis remains a major therapeutic complication of thalassemia major management, resulting in progressive organ dysfunction and death in a majority of patients. Although improved use of iron chelating agents and early splenectomy may improve iron balance in some patients, the goal of consistent long-term negative iron balance has yet to be achieved.

Preliminary animal studies using young red cells isolated on arabinogalactan gradients demonstrated that these cells had an improved in vivo survival compared to the survival of unselected whole blood when transfused into recipient animals. Since every 1 ml of red cells deposits 1.08 mg of iron in tissues at the end of the cells’ lifespan, selective transfusion with young cells with prolonged survival could theoretically increase the ratio of physiologic benefit to iron deposition. Standard blood for transfusion has an estimated mean cell age of 60 days, with an ideal residual mean survival of 60 days. In practice, transfused red cells generally survive less than 60 days. If units of red cells having a mean cell age of 30 days with a potential 90-day survival could be isolated and transfused, theoretically the transfusion requirement and amount of iron deposition could be halved.

Recently, Propper et al. reported preliminary data on the improved survival of young red cells isolated with a continuous flow blood cell separator. Because this technology is immediately feasible for human use and due to the limited technical information available about the method, we have examined this modality at greater length. Adequate quantities of red blood cells with prolonged in vivo survival (neocytes) can be prepared by modification of continuous flow blood cell separator component systems. Our initial experience with this technique is limited to thalassemia major patients, whose established transfusion requirements provided a basis for comparison with existing regimens. If successful, however, this therapeutic approach could be applicable to other patients with severe chronic anemia. The methodology and biologic properties of this transfusion product are the subject of the present report.

Materials and Methods

Blood donors were recruited from the donor files of the National Institutes of Health Clinical Center Blood Bank. Informed consent...
Samples were obtained through the platelet port, white cell port, similar operating conditions were during granulocyte collection that reticulocyte enrichment occurred siofl (28 U/mm) throughout the collection. Based on the observation Separator (IBM Corporation Princeton, N.J.) was adapted to this purpose.

Operating Conditions of the Aminco System

Donors were anticoagulated with a constant heparin-saline infusion (28 U/min) throughout the collection. Based on the observation during granulocyte collection that reticulocyte enrichment occurred in the red cell fraction immediately underlying the buffy coat, similar operating conditions were adopted for neocyte collection. Rotor speed was held constant at 700 rpm to give a G<sub>max</sub> of 41 g. Samples were obtained through the platelet port, white cell port, and from various positions of the red cell layer by manipulation of the interface position (Fig. 1A). Total flow rate from the donor was maintained at 60 ml/min with the harvest rate of young red cells kept constant at 5 ml/min. During the initial phase of experimentation, a variety of maneuvers were carried out to maximize the yield of young red cells, these are described below.

Operating Conditions of the IBM 2997

Donors were anticoagulated with ACD-A:1 vol to 13 vol of blood. The rotor speed was maintained at 650 rpm with a total donor flow rate of 60 ml/min. A single-stage disposable pathway (IBM Corporation, Princeton, N.J.) was used throughout (Fig. 1B). Young red cells were harvested at 5 ml/min. The hemoglobin content of the rotor eluate was monitored by the interposition of an external optical density monitor calibrated to hemoglobin concentration so that the operator could easily control the hemoglobin content of the neocyte harvesting line.

Routine Hematologic Tests

Hemoglobin concentration was measured as cyanomethemoglobin at 540 nm by a standard technique. White cell counts and platelet counts were performed as previously described. Reticulocyte counts were performed on thin smears after new methylene blue staining. One thousand cells were counted under 1000x oil immersion and the percent reticulocytes calculated.

Calculation of Yields

Red cell yield for "neocyte" units and standard frozen red cell units were calculated on the basis of total hemoglobin content. A standard fresh whole blood unit contains 450 ml with a hemoglobin concentration of 14.0 g/dl to give a total hemoglobin content of 63 g. This method of expression allows for more uniform determination of yields rather than estimation of packed cell volumes.

Estimation of Mean Cell Age

Aliquots of donor whole blood and fractions obtained from the blood cell separators were freed of leukocytes and platelets by filtration through α-cellulose-microcrystalline cellulose. Pyruvate kinase (PK) activity was measured as previously described. Based on probit plot analysis, mean cell age was estimated by use of the established relationship between an age-dependent enzyme (PK) and red cell buoyant density distribution. The following equation was obtained to express the relationship between mean cell age and pyruvate kinase activity: y = 1.7258 - 0.147x, where y = log<sub>10</sub> pyruvate kinase activity and x = the probit of a given cell aliquot derived from a cumulative frequency distribution analysis of red cell buoyant density distribution for a population of normal subjects. Red cell PK activity for unfractonated cells is normally distributed about a mean value of 9.97 U in our laboratory. The unfractonated red cell activity of each donor was assigned a value of 1.0, and subsequent cell fraction PK activity was expressed as a ratio relative to that value. Unfractonated red cells have a PK value of 1.0 and a theoretical mean cell age of 60 days with a probit value of 5.0. Based on this model, a relationship between mean cell age and the ratio of PK activity for cells of different postulated mean ages could be constructed relative to unfractonated cells (Fig. 2). This model could then be used to derive an approximate mean cell age for a given cell aliquot. These predicted estimates were later correlated with in vivo measurements of radiochromium red cell survivals.

Measurement of Red Cell Survival

Sterile aliquots obtained from standard frozen red cell donor units and from frozen neocyte units were labeled with radiochromium
Fig. 2. Relationship between pyruvate kinase activity and mean cell age. Ordinate: PK activity of a given neocyte fraction is expressed as a ratio to unfractionated red cell PK activity. Abscissa: mean cell age in days expressed in the probit distribution.

after deglycerolization immediately prior to infusion. Red cell survival was expressed as the half-life calculated from regression analysis for the equation $N_t = N_0 (1 - t/T) e^{-kt}$, where $N_t$ is the number of cells surviving to time $t$, $N_0$ is the number of cells administered, and $k_e = 0.007$, the first-order rate constant for chromium elution. Predicted half-life was derived from the estimate of cell age based on the PK ratio for a given cell aliquot compared to whole blood (Fig. 2) by the expression $T_{50} = (120 - A_t) x 1/2$, where $T_{50}$ = predicted half-life, $120$ = theoretical lifespan for red cells, and $A_t$ = estimated mean cell age of the cohort. Early cell loss was measured by extrapolating the red cell survival curves to zero time using data from day 1 and later to construct the curve. The extrapolated value at $t = 0$ was compared to the observed value for circulating radioactivity at 30 min postinfusion to arrive at an estimate of early cell loss.

Preparation and Storage of Red Cells

Young red cells isolated from either continuous flow system were concentrated by centrifugation and prepared for frozen storage by standard technique. After deglycerolization prior to infusion, the final product was filtered in series through standard $170 \mu$ and $40 \mu$ filters.

RESULTS

Aminco Celltrifuge

Initial experience with this instrument had demonstrated that a rotor speed of 700 rpm produced a stable interface with optimal reticulocyte enrichment in the red cell zone beneath the buffy coat (Fig. 1A). It also became apparent that total flow rate (plasma pump rate plus red cell pump rate plus white cell pump rate) was critically related to the rate at which young cells could be harvested. A total flow rate of 60 ml/min was acceptable to most donors, particularly when a 150-ml reservoir was interposed between the donor and the rotor. Few donors could tolerate more rapid phlebotomy without collapse of the antecubital vein. The rate of extraction of young red cells was optimal at 5 ml/min. At more rapid collection rates, the yield of young cells declined sharply.

Two objectives in harvesting young cells were identified: (1) to obtain a transfusion product significantly enriched with young red cells and (2) to collect a sufficient amount of hemoglobin to make transfusion of the product clinically useful. It was decided to attempt to isolate red cells with a mean age of 30 days. Based on the observation that reticulocytes rise to the top of the red cell layer, the initial approach was to isolate the upper one-half of the red cell layer. Sampling for red cells at various depths across the platelet layer, white cell layer, and red cell layer demonstrated that neocyte enrichment decreased rapidly at deeper layers in the red cell zone (Fig. 3). Enrichment for young cells was excellent in the platelet and leukocyte zones. However, the quantity of red cells in the first two areas was extremely small.
Because hemoglobin content of the leukocyte region averaged between 3 and 5 g/dl and consisted largely of young cells, it was decided to collect at the leukocyte port and to enrich this layer with red cells in order to determine the most effective hemoglobin concentration at which maximal yields of hemoglobin with an adequate young mean cell age could be achieved. At hemoglobin concentrations below 3 g/dl, a group of five donors consistently yielded red cells with a PK activity ratio ≥1.3, having an estimated mean cell age of 30 days (Fig. 4A). As hemoglobin concentration increased, PK activity declined (Fig. 4A) parallel to the decline in reticulocyte count (Fig. 4B). As a result of this experience, neocytes were collected under the following conditions: rotor speed 700 rpm, total pump speed 60 ml/min, neocyte harvest rate 5 ml/min, and line hemoglobin content 3 g/dl. The latter was regulated by periodic monitoring of the outflow line via a 3-way stopcock. These conditions would theoretically produce 9 g of hemoglobin/hr, which after a 4-hr collection period would net approximately 36 g of hemoglobin with a mean cell age of 30 days. The total hemoglobin yield is about two-thirds of a standard frozen red cell unit.

Cumulative experience over a 6-mo period (Table 1) demonstrated that an average hemoglobin yield of 30.4 g could be achieved and that an average estimated mean cell age of 35 days compared to 60 days for unfractionated blood was obtainable. These data include the total experience during the experimental period. Some units were of poor quality or low hemoglobin content. These failures could usually be traced to early operational problems that were corrected as operating experience increased. The most frequent problems were interface instability and poor hemoglobin concentration control.

**IBM 2997**

When the IBM 2997 continuous flow blood cell separator became available, this system was evaluated for neocyte collections. Initial experience with rotor speed, flow rates, and harvest rates based on our earlier experience with the Aminco Celltrifuge led to the following operating conditions: rotor speed 650 rpm, total flow rate 60 ml/min, and neocyte harvest rate 5 ml/min. Analogous experiments looking at hemoglobin-neocyte enrichment of the leukocyte port (Fig. 5A and B) demonstrated that hemoglobin concentrations as high as 4.0 g/dl would still result in adequate enrichment. Under these conditions, a total hemoglobin collection of about 48 g could be achieved in a 4-hr donation period. Cumulative experience with this system over a 6-mo period (Table 1) resulted in an average yield of 36.4 g of hemoglobin having an average mean age of 30 days and an average reticulocyte count of 4.8%. The addition of an in-line continuous reading hemoglobinometer greatly facilitated later procedures and has resulted in an improved average hemoglobin collection of 42.3 ± 1.6 g.

Failure to achieve the goal of 48 g is in part due to inclusion of early poor runs in the data sample and problems with donor intolerance to citrate in the last hour of collection. This has been resolved by modifying

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**Table 1. Hemoglobin Content, Reticulocyte Count, and Estimated Mean Cell Age of Units Collected on Aminco and IBM Systems**

<table>
<thead>
<tr>
<th>System</th>
<th>Donor</th>
<th>Hb Yield (g)</th>
<th>Reticulocyte Count (%)</th>
<th>Estimated Age (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminco</td>
<td>52</td>
<td>30.4</td>
<td>5.0</td>
<td>35</td>
</tr>
<tr>
<td>IBM</td>
<td>45</td>
<td>36.4*</td>
<td>4.8</td>
<td>30</td>
</tr>
</tbody>
</table>

*Data obtained before use of in-line hemoglobin monitor.
†n, number of donor collections.
the anticoagulation regimen. At the start of each collection, 1000 U of heparin are infused into the withdrawal and return veins in a single bolus. The donor is then anticoagulated during the procedure with ACD-B at the same ratio as for ACD-A. This has reduced citrate toxicity from 20% of donors to less than 2%. Slowing the flow rate to 50 ml/min with a rotor speed of 550 rpm has also contributed to decreased citrate toxicity.

In Vivo Survival of Neocytes

To establish the principle that neocytes could have a vastly improved survival, narrow cohorts of young cells were isolated from three normal eusplenic donors, labeled with radiochromium, reinfused, and their survival measured (Fig. 6). Average estimated mean cell age was 6.5 days, and the average half-life observed was 57.2 days. There was a prolonged survival in each case with a tendency to plateau after a slightly more rapid initial slope. There was no evidence for accelerated cell loss after the first 25 days, indicating that the cells were composed of a uniformly younger cell population.

Routinely prepared standard frozen red cells infused into 7 asplenic beta thalassemia major patients had an average radiochromium half-life of 29.5 days ± 3.6 (SEM). This was compared to an average half-life of 47.4 days ± 2.3 for 8 neocyte units infused into the
same 7 patients (Table 2). The predicted half-life based on PK activity averaged 49.5 days ± 1.3 and was not significantly different from the observed radiochromium value by paired t test, p < 0.3. The average hemoglobin content for the labeled neocyte units was 42.3 g ± 1.6. Early cell loss for the labeled neocyte units averaged 6.4% ± 2.4%. There were no adverse reactions observed in the 7 patients.

One subject had two survivals that were discordant with the predicted value (indicated by asterisk in Table 2). This patient had also shown a very short survival with regular frozen red blood cells (T½ 16 days). We were unable to demonstrate any alloantibodies in this individual to account for this phenomenon.

**Effect of Freeze Preservation on Neocyte Units**

Reticulocyte counts of processed neocyte units (3.7% ± 0.2%) sampled after thawing were lower compared to those of fresh units (4.3% ± 0.2%) sampled at the time of collection. This difference was significant by paired t test, p < 0.001. In a similar fashion, PK activity also decreased when thawed final product was compared to the freshly isolated cells: 13.2 U ± 0.5 versus 14.4 U ± 0.8, p < 0.02. There was poor correlation (r = +0.42) between reticulocyte count and PK activity of neocyte units, and there was no significant correlation between either the initial PK activity and the change in PK activity with freezing, the initial reticulocyte count and the change in reticulocyte count with freezing, nor between the change in PK activity with the change in reticulocyte count. Thus, while there was a slight reduction in enzyme activity with the freeze-thaw process, this could not be attributed solely to the selective destruction of reticulocytes. Although reticulocytes are still recognizable by supravital staining in thawed blood, it is not known if they have a normal survival. The reticulocyte count of freshly collected neocyte units did not correlate closely with observed radiochromium survival of the final thawed and processed product (Table 2).

**DISCUSSION**

Maintenance of adequate hemoglobin levels and negative iron balance has been achieved in older thalassemia major patients using hypertransfusion regimens combined with continuous chelator infusion.15 Graziano et al.4 have recently shown that splenectomy also reduces the transfusion requirement of the older thalassemia major patient. As of this time, none of these modalities has been shown to be capable of preventing the development of hemochromatosis in the very young patient. Ultimately, prevention of early iron accumulation, when chelators are less effective, may be more important in long-term management than effecting net negative iron balance in the already overloaded individual.

The current study explores a new approach to the therapy of chronic transfusion-dependent patients. It demonstrates that it is possible to isolate normal young red cells that have an enhanced survival when transfused into asplenic patients. The final average hemoglobin yield in completed IBM neocyte units ready to transfuse was 35.5 g/U. The average yield for a standard frozen red cell unit is 51 g in our blood bank, thus a neocyte unit is 70% of a regular unit. Although a neocyte unit is smaller than a standard unit, adequate quantities of young red cells are obtainable, and with the use of a limited donor pool, the risk of multiple donor exposure could be further reduced.

Survival studies in both eusplenic normals and asplenic patients suggest that it is not enrichment for reticulocytes alone that accounts for the increased lifespan of these cells. The neocyte units appear to consist of true cohorts of younger red cells including cells beyond the reticulocyte stage. Although differential rates of 51Cr elution might account for some of the enhanced survival, little data are available to compare the 51Cr elution rate from young and old cells. In rabbits, the elution rate is constant at 2.7%/day for the initial 70% of the cells’ lifespan, and then it increases by 1.5% for the residual lifespan.5 Cline and Berlin observed a range of 51Cr elution rates for humans from 0.62% to 2.7% per day, but for any one subject a constant rate was observed and there was no correlation between the elution rate and measured red cell lifespan.6 We have used a single elution rate of 0.7%/day for all studies based on fitting of survival curves for routine frozen red cells. This rate is at the lower end of the reported range and would minimize the amount of over correction applied to young cells. It was derived from unfractionated cells and since in the rabbit model significant differences in elution rate...
were only seen with the very oldest cells, the degree of overestimation would be further minimized. Assuming that the application of too high an elution rate to the younger cell units was used, this still could not account for the large survival differences observed. There is also evidence that $^{51}$Cr uptake by young and old cells is equivalent, which would rule out differential binding as a contributing factor.\(^7\)

We have used a model based on the relationship between cellular buoyant density and PK activity to provide a convenient estimate of mean cell age. This is based on earlier work that established a relationship between cell age and buoyant density.\(^6\) The present adaptation of this model to estimate neocyte unit mean cell ages is dependent on the assumption that these are relatively narrow cohorts with respect to age. These cohorts are probably composed of overlapping groups of cells each with slightly different means ages and, as such, the model only provides an approximate estimate of age. The agreement between the age estimates and observed in vivo age is reasonable. We have performed survival studies of red cells from suboptimal neocyte units in several patients and found good agreement with the predicted survival for this product as well. An additional value of the age prediction is its use to control quality in order to avoid transfusing units not sufficiently enriched for young cells. As evidenced by the total collection data, there are some donors who provide relatively poor neocyte enrichment and thus would not give enhanced survival. We have used this methodology to exclude these potentially “nonbeneficial” units from already iron-overloaded patients.

Neocyte units are also enriched with leukocytes and platelets. The average leukocyte content in a random sampling of these units prior to freeze preservation was 196.5 x $10^5$ cells/U. The average platelet count prior to freezing was 400 x $10^9$/µl. Differential smear examination revealed 84% of these leukocytes to be lymphoid. Transfusion of relatively long-lived lymphoid cells could be a potential problem; however, sampling of neocyte units after thawing the cell washing at the time of transfusion reveals an average leukocyte count of 2.6 x $10^9$ cells/U with only a rare intact lymphoid cell demonstrated on these smears. Thus, 99% of the leukocytes are lost during the freeze–thaw process and washing of the units prior to transfusion should remove soluble nuclear debris. No platelets were found after deglycerolization and washing. In spite of these precautions, the inadvertent transfusion of soluble DNA remains a potential problem. We are currently monitoring the patients’ posttransfusion circulating DNA levels and prospectively assaying patients for the development of anti-DNA antibodies.

Neocyte collection is an expensive and time-consuming procedure. The average cost per neocyte collection is approximately $300 compared to $68 for standard frozen red cells. In addition, a pool of highly motivated donors is required to support the transfusion program, although donor acceptance of the procedure has been good in spite of the long collection time. The initial problems with mild citrate toxicity have been resolved by use of ACD-B instead of ACD-A, and the currently used one-time heparin flush has not been associated with any toxicity.

Six of the seven patients have had an improved red cell survival with neocytes, one patient had a short survival on two occasions. This patient has also had short survivals with standard frozen blood. This may have been due to individual defective units or to the use of the radiochromium survival, a very sensitive technique for detecting alloimmunization. Use of such a sensitive technique to measure in vivo compatibility may detect minor blood group incompatibilities that are not obvious in routine screening and crossmatching of these multiply-transfused patients. There may also be other factors in these chronically ill patients, which could lead to shortened red cell survivals; however, to date we have observed this discrepant survival in only one patient.

Transfusion with selectively isolated young red cells appears to be a promising approach to the management of patients with transfusion-dependent chronic anemia. It may be possible to avoid the long-term development of hemochromatosis while maintaining near normal hemoglobin levels which would allow patients to lead more normal lives. We have as yet to demonstrate a sustained clinical benefit for this technology that will require at least a 1-yr trial to show a convincing decrease in blood use. Efforts are currently underway in our laboratory and others to simplify the procedure and to reduce costs and technical difficulties in order to make this therapy more widely available.

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