A Simple Technique for Preparation of Young Red Cells for Transfusion From Ordinary Blood Units

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We have developed a rapid and simple method for the isolation of relatively young cells from ordinary fresh or previously frozen blood units, using the IBM-2991 Blood Cell Processor. Based on in vitro measurements of red cell pyruvate kinase activity, the resolution of the method is excellent, comparable to that of the stractan discontinuous gradient system. In splenectomized patients with thalassemia major, the administration of the "youngest" 50% of blood units yielded $^{51}$Cr survival times of 40 and 42 days for fresh packed red cells and 41 and 43 days for previously frozen red cells.

As the circulating erythrocyte goes through its 120-day lifespan, it becomes increasingly smaller and more dense with time. These age-dependent size and density changes have led to the development of several laboratory techniques capable of separating red cells according to age.1-4 Probably the simplest and most widely used technique is the stractan (arabinogalactane) discontinuous density gradient,5,6 which has been used extensively to study the aging process of normal7 and genetically abnormal erythrocytes.8,9 In 1973, Corash and coworkers2 proposed that it might be feasible to isolate on stractan gradients relatively young erythrocytes for transfusion into patients with thalassemia major or other dyserythropoietic anemias, and thereby increase the interval between blood transfusions and decrease the rate of iron accumulation. Subsequent studies in rabbits of the in vivo survival of cohorts of erythrocytes isolated by this technique demonstrated significantly prolonged survival of the top less dense fractions, when compared to unfractionated blood or the bottom more dense fractions.10 However, because stractan is potentially antigenic, it does not seem to be ideal for human use. Recently, continuous flow blood cell separators, which also utilize differences in cell buoyant densities to isolate specific blood cell types for transfusion, have been adapted to collect very young red cells.5,6

We report here the development of a simple, rapid, and relatively inexpensive method for the preparation of young erythrocytes from ordinary blood units using the IBM-2991 Blood Cell Processor.

MATERIALS AND METHODS

Units of whole blood were collected from eight random donors in citrate-phosphate-dextrose (CPD) containing bags (Fenwal, Deerfield, Ill.) and fractionated with the IBM-2991 Cell Processor within 24 hr of collection as described below. An additional 10 ml of blood was collected into a syringe and defibrinated with glass beads for a parallel stractan density gradient separation.

Once it was determined that whole blood units could be fractionated in an age-dependent manner with the IBM-2991 Cell Processor, it became important to determine whether age-dependent separation could be achieved after freeze-thawing, since most chronically transfused patients ultimately require frozen blood. Six units of packed red cells were frozen by a low-glycerol rapid freeze process,11 deglycerolized manually at the New York Blood Center, and immediately fractionated according to the method described below. An additional 6 units of packed red cells were frozen by a high-glycerol, mechanical refrigeration method12 in our laboratory, deglycerolized in the IBM-2991,11 and fractionated as described below.

Cell Separation

The IBM-2991 Cell Processor is a centrifuge that was originally designed to remove the preservative agent from a unit of frozen-thawed red blood cells, or wash a unit of fresh blood. In these studies, units of blood were introduced into the instrument's processing bag, and the remaining space filled with saline. The blood was then spun at 3000 rpm for 5 min, and the supernatant removed at a flow rate of 450 ml/min. The cells were subsequently washed twice with resuspending in saline, followed by agitation for 1 min, centrifugation at 3000 rpm for 3 min, and removal of the supernatant at a flow rate of 450 ml/min. The supernatant was driven out of the port in the center of the processing bag by a "superout" pump that compresses the bag while spinning. The tubing from the center port passes through a detector, which disengages the pump when triggered by red cells.

The critical step required for an age-dependent cell fractionation occurs at the end of the second wash cycle. (For previously frozen blood units being deglycerolized in the IBM-2991, this critical step was initiated after the final wash with 0.9% saline containing 0.2% dextrose.) As the red cell boundary reaches the center port, but before the red cell detector is tripped, the cells were allowed to continue spinning at 3000 rpm for 10 min (by pushing the "hold"

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At the end of this 10-min spin, the superout pump was reengaged at a reduced flow rate of 60 ml/min (the instrument must be calibrated manually for this flow rate). After disengaging the red cell detector, red cells were slowly expressed through the center port, youngest cells first, in an age-dependent sequence. For the purpose of the in vitro pyruvate kinase (PK) studies, red cells were collected in 35-ml fractions. An aliquot of each fraction was filtered through α-cellulose-microcrystalline cellulose columns to remove contaminating leukocytes, washed 3 times with buffered saline, and subsequently assayed for pyruvate kinase (PK) activity.7

The activity of PK in the red cell is known to decline as a function of cell age.8 We have therefore used PK activity as a marker of red cell age. When, for each red cell fraction in a gradient, the logarithm of cell age is plotted against its probit (derived from the cumulative distribution of red cells in the gradient), a slope of decline in PK activity is defined. By converting the probit axis into cell age, from 0 to 120 days, a half-life of red cell PK activity can be calculated.8 Using fresh blood in the high-resolution arabinogalactan (stractan) density gradient system, a slope value of −0.14 has been obtained, from which a red cell PK half-life of 29 days was derived.8 In order to compare the resolution of age-dependent cell separation obtained with the IBM-2991 to that obtained by the stractan gradient system in a quantitative fashion, we have similarly measured slopes of decline of PK activity in consecutive fractions. Using this model, the smaller the slope in comparison to that obtained by the stractan method, the poorer the resolution of age-dependent separation.

Measurement of Red Cell Survival

In vivo survival of cohorts of red cells labeled with radiochromium13 was determined in six splenectomized patients with thalassemia major after obtaining appropriate informed consent. Cohorts of red cells were mixed with the patient's plasma to a hematocrit of approximately 50% of whole blood units, determined as 50% of the red cell mass by weight; red cells were collected into a transfer-pack that had been preweighed and placed on a balance adjacent to the IBM-2991 centrifuge. One patient received a labeled cohort of cells from the “top” 30% of a whole blood unit, while another control patient received cells that were sham-separated, but remixed prior to radiolabeling. Two additional patients received cells from the “top” 30% of blood units, which were previously frozen by the high-glycerol technique13 and subsequently deglycerolized and fractionated in a single operation of the IBM-2991.

RESULTS

The composite exponential decline in the pyruvate kinase (PK) activities of red cell fractions obtained from six discontinuous stractan gradients is shown in Fig. 1A. The rate of decline of PK activity of 35-ml fractions of packed red cells obtained from the IBM-2991 is shown in Fig. 1B. These slopes were so close as to indicate that the resolution of age-dependent cell separation by these two techniques is essentially identical. Similar results have been obtained when units of packed red cells, rather than whole blood, have been used as the starting material.

The resolution of age-dependent red cell separation that can be achieved with the IBM-2991 is visually demonstrated in Fig. 2. A unit of blood was fractionated as above into 50-ml fractions. Samples from the first 50 ml and the last 50 ml of red cells expressed from the processing bag were subsequently centrifuged on stractan density gradients. The density distribution of the first 50 ml fraction clearly consists of red cells that are relatively less dense than the red cells contained in the bottom 50 ml.

From a practical viewpoint, since many transfusion-dependent patients require frozen-thawed erythrocytes...
to minimize the incidence of leukocyte reactions, it would be useful if young red cells could be prepared after deglycerolization. Therefore, six units of blood were frozen by a low-glycerol technique,\textsuperscript{11} deglycerolized manually, and subsequently fractionated immediately as above. In these cases, the IBM-2991 young-cell fractionation method was incapable of achieving a good resolution of age-dependent cell separation. Erythrocyte pyruvate kinase activity declined, but at much slower rates, than in fresh blood, in consecutive fractions of deglycerolized red cells, using either the stractan (slope $-0.10$) or the IBM-2991 (slope $-0.07$) method.

However, when the same experiment was repeated using 6 units of blood that had been frozen by a high-glycerol technique\textsuperscript{12} and deglycerolized using the IBM-2991,\textsuperscript{13} a better age-dependent cell separation was achieved with both the stractan (slope $-0.13$) and IBM-2991 (slope $-0.11$) methods. Although the slopes of PK decline were significantly different from each other and from those obtained from fresh blood, the differences among the slopes was small. Thus, it would appear that deglycerolization and young cell fractionation can be achieved in a single operation of the IBM-2991 when a high-glycerol freezing technique is used, with a resolution of cell separation only slightly poorer than that achieved with fresh blood.

\textit{In Vivo Red Cell Survival}

Packed red cells that were sham-fractionated but remixed prior to radiolabeling yielded a radiochromium half-life of 29 days in a splenectomized patient with thalassemia major, agreeing with the reported survival of ordinary blood units previously reported by others.\textsuperscript{5,6} In two patients who received the youngest, or “top” 50% of whole blood units, radiochromium half-lives of 40 and 42 days were obtained. In one patient who received the “top” 30% of a whole blood unit, a radiochromium half-life of 56 days was obtained. Finally, in two patients who received the “top” 50% of blood units that were previously frozen by the high-glycerol technique,\textsuperscript{12} radiochromium survivals of 41 and 43 days were obtained.

\section*{DISCUSSION}

The chronic administration of relatively young red cells would improve the management of chronically transfused patients by decreasing the frequency of transfusion and the rate of iron loading. To date, efforts to prepare young red cells for transfusion have centered around the use of the Aminco\textsuperscript{5} and IBM-2997\textsuperscript{4} continuous flow blood cell separators. Young cells obtained in such a manner show improved survival when transfused into patients with $\beta$-thalassemia major.\textsuperscript{5,6} In practical terms, however, the pheresis approach to young cell collection is extremely expensive, requires about 4 hr of a blood donor’s time, and is associated with some risk to the donor. For these reasons, it appears unlikely to enter widespread clinical use, despite its theoretical advantage of obtaining extremely young red cells.

We have therefore focused our efforts on the development of a more practical technique that could isolate the youngest red cells from ordinary units of blood. By systematically altering the centrifuge speed, centrifugation time, and flow rate of the IBM-2991, we have been successful in developing a method capable of achieving age-dependent (presumably density-dependent) cell separation. As evidenced by in vitro pyruvate kinase studies, the resolution of the method is excellent, achieving results similar to cell separation on stractan discontinuous gradients. The red cell survival studies completed to date have confirmed the conclusions based on in vitro pyruvate kinase studies\textsuperscript{16} that this method is capable of achieving age-dependent red cell separation.

Our observation that, following high-glycerol freezing,\textsuperscript{12} the IBM-2991 can be used to deglycerolize and fractionate young cells in one operation, albeit with slightly poorer resolution than that achieved in fractionating fresh blood units, is an important one because it might simplify the possibility of providing young cells on a widespread routine basis. In the United States, most chronically transfused patients with thalassemia and other dyserythropoietic anemias are already supported with frozen-deglycerolized red cells. By adding a 10-min centrifugation step to the deglycer-
eralization procedure, "half-units" of young red cells for transfusion could be prepared in virtually any blood bank at a cost comparable to the cost of a unit of frozen blood. While the cost of two half-units of previously frozen red cells would be approximately twice that of an ordinary unit of frozen red cells, the net cost of blood transfusions should increase by only 40%, since blood consumption should decrease by approximately 30%. By comparison, Corash and coworkers have estimated that the cost (at the NIH) of preparing young red cells for transfusion by the pheresis method is three times that of typical frozen blood costs. The increased cost of young half-units of blood may ultimately be offset by a decrease in the frequency of hospital visits, clinic fees, days missed from work, and transportation costs.

We do not consider these data to imply that young red cell preparation by this technique should immediately become a routine blood bank procedure for all transfusion-dependent patients. A long-term clinical trial is in progress in our clinic that will accurately quantitate the reduction in the annual blood requirement and iron load in groups of patients in whom these parameters have carefully been established. The reduction in hospital visits and the actual cost of young red cell transfusion therapy will also be determined. Until the actual benefits and costs of such therapy are known, we feel that widespread use of young red cell transfusions may unnecessarily add to the financial costs of a disease that is already a financial burden to most families. The clinical usefulness of the "oldest" 50% of blood units must also be evaluated so as to minimize the potential waste of blood. Nevertheless, we are optimistic that the potential increased costs of health care may be offset by fewer transfusion-related disruptions in day-to-day life and by a significant extension of life in patients with thalassemia major.

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