Separation of younger red cells with improved survival in vivo: An approach to chronic transfusion therapy

(thalassemia/iron overload/buoyant density)

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ABSTRACT Transfusion of donor blood containing predominantly younger red cells with prolonged survival in vivo could significantly reduce the iron overload of patients requiring chronic transfusion. Age-dependent separation of red cells can be obtained by buoyant density centrifugation on isotonic solutions of arabino-galactane. By this technique, rabbit red cells were separated on a single layer of arabino-galactane and the appropriate fraction, after being labeled with 51Cr, was reinjected into the donor. The survival in vivo was calculated by a mathematical model which corrects for both 51Cr elution and random loss. There was a significant difference in survival in vivo between the light young red cells and the heavy old red cells. The potential survival in vivo of the 50% lightest red cells was 56 days, compared to 25 days for the heaviest red cells. Arabino-galactane appeared to be devoid of acute toxicity and of strong antigenicity and it did not appear to adhere to the red cell stroma. These data extrapolated to humans indicate that it may be feasible and advantageous to use red cells fractionated by this technique for transfusion. The 50% lightest human red cells can be expected to have a mean survival of 88 days, compared with 60 days for unfractionated blood. Transfusion of young red cells could significantly reduce the iron overload for patients requiring chronic transfusion, by avoiding infusion of the oldest red cells, which contribute equally to iron overload yet offer only transient survival in vivo.

Transfused red cells removed from the circulation at the end of their life span are the source of iron overload in patients with chronic anemias, who depend on repeated transfusion for survival. Excess iron is accumulated by patients with thalassemia major, for instance, in such large amounts that this disorder may presently be viewed primarily as a problem of iron overload (1). Human blood contains equal proportions of red cells of ages between 0 and 120 days; the iron content (108 mg/dl) is the same regardless of cell age. Thus the contribution to iron overload is the same from the oldest transfused red cells, which survive only a few hours, as from the youngest ones, which circulate for months. Obviously, transfusion would be more effective in terms of the ratio of physiological benefit to iron overload if the oldest red cells were removed and only the younger cells administered. Recent advances in the technique of cell separation (2, 3) make it appear that this goal is now feasible. Using an animal model, the present study evaluates the effectiveness of a simplified density gradient separation technique in yielding cells with improved survival in vivo in quantities suitable for transfusion. In order for this procedure to be of practical value, it is necessary to obtain at least 45–55% of the original blood unit. This approach to chronic transfusion may significantly reduce the iron overload.

MATERIALS AND METHODS

Animals. White New Zealand rabbits (3–4 kg) and guinea pigs (530–670 g) were individually caged. Swiss CD mice (20–25 g) were housed six per cage. All animals were fed standard laboratory diets.

Arabino-Galactane. Solutions were prepared as described (4). Two solutions of 1.080 and 1.100 specific gravity (at 20°) were initially prepared and mixed to yield solutions of the desired specific gravity. The precise concentration of each arabino-galactane solution was measured by refractometry and, if necessary, was adjusted.

Density Separations. Three milliliters whole blood were layered over the appropriate solution of arabino-galactane in a 1.58 X 10.16 cm polycarbonate tube. The tube contents were covered with mineral oil, then centrifuged at 20,000 rpm (51,900 x g at center of tube) for 15 min in the SW-27 rotor in a Spincos model L3-50 ultracentrifuge. After centrifugation, the tube was first sliced just above the upper red cell layer to remove the mineral oil, then at the center of the clear arabino-galactane layer to separate the lighter from the heavier red cell layers. The relative percent of the red cells in each layer was estimated by measurement of the hemoglobin concentration (5), after both layers were brought to the same volume with buffered saline/glucose (pH 7.4, 291 milliosmoles/liter: 8.1 g of NaCl, 1.22 g of Na2HPO4 0.219 g of NaH2PO4 2H2O, and 2 g of glucose to 1000 ml with H2O). The red cells were then washed three times with buffered saline/glucose. Either the lighter or the heavier red cells or both were separately labeled with 51Cr, then washed and ultimately remixed with the remaining unlabeled red cells before reinfusion into the donor animal through the marginal ear vein.

51Cr Labeling. Washed red cells were incubated with 50 μCi of Na251CrO4 (New England Nuclear, specific activity > 20 μCi/μg of Cr) for 15 min at 37°. The excess unbound 51Cr (usually less than 5% of total) was removed by five washes with buffered saline/glucose.

Blood. Samples were obtained from the central ear artery 15 min after injection (called the initial sample), 24 hr later; then three times weekly for at least 60 days. Each sample consisted of 0.5 ml collected in a syringe wetted with a drop of heparin. The total amount of blood removed was <5% of the total blood volume. Hematocrits remained constant throughout the experiment.

Radioactivity Measurements. Two-tenths milliliter of whole blood was pipetted at the bottom of a counting vial of 10-mm diameter containing 1 ml of distilled water. The radioactivity of all samples from the same animal was measured simultaneously at the end of the experiment to avoid correction for

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physical decay. At least 10,000 counts were obtained for each sample, using a Packard AutoGamma scintillation counter equipped with additional 3-inch (7.6 cm) lead shielding (background 10 cpn).

Measurement of Red Cell Survival. The radioactivity of all samples was divided by that of the respective initial sample. The resulting ratios obtained between days 5 and 15 were used to derive, by exponential least-squares regression, the computed ratio at time 0. The difference between 1 and the computed ratio at time 0 represents the "early loss." For further computations, all ratios were divided by the computed ratio at time 0 to normalize for "early loss."

Toxicity Studies of Arabinogalactane. Groups of six mice each were injected with progressive doses of arabinogalactane, between 4 and 22 g/kg, using either the crude material (Stractan II) dissolved in water or the purified material, after passage through Amberlite MB-3 column and adjustment of the pH and osmolarity as described (4).

Antigenicity Tests. Four guinea pigs were presensitized with intraperitoneal injections of 0, 10, 100, or 1000 mg/kg, followed by challenge 21 days later with an intravenous injection of 10 mg test material per kg.

Estimation of Residual Arabinogalactane. Arabinose (a sugar absent in the red cells) was measured by gas chromatography of the trimethylsilyl sugar derivatives by the method of Sweeley et al. (6). Red cell stroma were prepared for this purpose as described by Gattono et al. (7).

RESULTS

Effect of Fractionation Procedure on Red Cell Survival. In three rabbits, red cells were fractionated into equal proportions of light and heavy; the two fractions were separately labeled with $^{51}$Cr and washed, then mixed together and reinfused into the donor. In three other rabbits, the red cells were labeled with $^{51}$Cr immediately after removal from the donor, then washed and reinfused. The difference in the rate of disappearance of radioactivity between these two groups of animals was essentially indistinguishable. The "early loss" in both experiments ranged from 0 to 6% (mean 3.6%). These findings indicate that the centrifugation on arabinogalactane and the inherent additional manipulations do not result in any alterations of the ability of the red cell to survive in vivo.

Comparison of Rates of Survival of Red Cells for Different Specific Gravities. Five groups of three rabbits each were compared. In group I, the blood was centrifuged on arabinogalactane of 1.086 specific gravity to obtain a very small top fraction (average 10%, range 8–12%); this was labeled with $^{51}$Cr and reinfused. In groups II, III, and IV, the blood was centrifuged on arabinogalactane of 1.092 specific gravity to obtain top and bottom fractions of approximately equal proportion (average 50%, range of the top fraction 42–58%). In group II the top fraction only was labeled with $^{51}$Cr and reinfused. In group III, both top and bottom fractions were separately labeled with $^{51}$Cr, washed, and mixed together before reinfusion. In group IV, the bottom fraction only was labeled with $^{51}$Cr and reinfused. In group V, the blood was centrifuged on arabinogalactane of 1.098 specific gravity to obtain a very small bottom fraction (average 10%, range 7–13%); this was labeled with $^{51}$Cr and reinfused. This experimental model provided four groups of animals in which the decline of radioactivity could be followed for, respectively, the top 10% (group I), the top 50% (group II), the bottom 50% (group IV), and the bottom 10% (group V), and compared to the rate of decline of radioactivity of the control ( sham fractionation) represented by group III.

There was a significant difference in the percent of early loss, which was 1.0% for group I, 3.0% for group II, 6.2% for group IV, and 11.1% for group V, compared with 3.6% in the control group (group III). These findings indicate that the "early loss" is the least in the lightest red cells and increases progressively with increasing red cell density. Since the early loss most likely reflects damage due to the manipulations inherent in the collection and labeling procedure, these findings indicate that the lightest red cells are the most resistant and the heaviest red cells the least resistant to this damage. Fig. 1 clearly shows that the rate of decline in radioactivity is the least for the top 10% of red cells and progressively increases with increasing cell density, the two fractions with the heaviest red cells having rates of decline of radioactivity significantly faster than the sham-fractionated red cells. These findings indicate that there is a progressive decrease in the survival in $\text{vivo}$ of red cells of increasing density, from the lightest to the heaviest. Of greatest significance, if this technique is to be applied to human transfusion, it is the distinctly better survival of the top 50% red cell fraction when compared to the sham-fractionated red cells; this is even more striking when compared to the bottom 50% red cell fraction. In two additional groups of three rabbits each, the survival of the top 25% and the bottom 25% of red cells were similarly estimated. The results (not shown) were essentially intermediate between those for the respective 10% and 50% fractions.

Analysis of $^{51}$Cr Survival Curves. Interpretation of the survival curves of red cells labeled with $^{51}$Cr is complex in rabbits, since their red cell life span is limited by a significant rate of random loss before the finite life span is reached, at which point a more rapid rate of loss due to intrinsic aging becomes prominent. Moreover, the rate of elution of $^{51}$Cr itself is unknown. From previous experiments from this laboratory, which used cohort labeling in vivo with the noneluting isotope [2-14C]glucose, it is possible to estimate the rate of random loss (0.9%/day), the end of the finite life span (50 days), and the faster rate of loss due to aging (5.71%/day) (2). With these parameters, the percent of the original cells present at the time of the labeling that is expected each day thereafter can be computed with the following formula (see Appendix):

![Figure 1](image-url)
where \( R \) = daily rate of random loss = 0.0099; \( A \) = daily rate of aging loss = 0.0571; \( E \) = end of finite life span = 50 days; \( t \) = time in days; and \( S \) = sum of all cells present at time \( t \). These expected values computed for unfractionated red cells are shown in the upper curve of Fig. 2A; the lower curve of Fig. 2A is the percent of radioactivity observed at corresponding days. The ratio of the expected to observed radioactivity is the result of \( ^{51}\text{Cr} \) elution (see in Fig. 2B). It is apparent that a constant rate of \( ^{51}\text{Cr} \) elution of 2.7%/day occurs for the first 35 days, followed thereafter by a modest increase to 4.2%/day. These data indicate that there is no marked increase in the rate of \( ^{51}\text{Cr} \) elution with red cell aging in rabbits. Therefore, the different rates of decline of circulating radioactivity between the lightest and the heaviest red cells reflect a real difference in survival rates. It is possible to compute the difference in survival of the light compared to the heavy cells that is due solely to aging loss by correcting each point for both \( ^{51}\text{Cr} \) elution and daily random loss, since both of these occur at a known and constant rate. These computations are shown in Fig. 3, which indicates that the lighter 50% of red cells survive in the circulation for almost the duration of their finite life span, while the heavier 50% initiate their aging loss almost immediately after reinfusion into the circulation. These data indicate that the potential mean life span of the 50% lighter rabbit red cells is nearly 56 days, while the potential mean survival of the 50% heavier red cells is only half this (the true mean survival, including random and aging loss, is obtained correcting only for \( ^{51}\text{Cr} \) elution; it is, respectively, 45 and 22 days). These results from the rabbit, a species with significant random red cell loss, can be extrapolated to the human, a species in whom random red cell loss is negligible. Assuming that human red cells can be separated on arabinogalactan with an equal degree of age dependency, the expected mean survival of the top and bottom 50% would be 88 days and 32 days, respectively, compared with the theoretical 60 days’ mean survival of unfractionated blood.

Toxicity and Antigenicity of Arabinogalactan. The mean lethal dose (LD\(_{\text{50}}\)) of crude solutions of Stractan II was greater than 22 g/kg. The LD\(_{\text{50}}\) of purified arabinogalactan was also in excess of 22 g/kg. There was no evidence of anaphylaxis in guinea pigs. These data indicate that arabinogalactan is devoid of acute toxicity and it is unlikely to be a strong sensitizing antigen.

Absence of Residual Arabinogalactan in Red Cell Stroma. By gas chromatography, complete absence of any arabinose peak was demonstrated in the stroma of red cells that had been exposed to arabinogalactan through centrifugation and separation procedures identical to those described above. These findings indicate that there is no permanent bond of arabinogalactan to the red cell membrane, and the only residue present in the cell fractions will be in the supernatant.

**DISCUSSION**

Separation of red cells according to age by density is based on the observation by Key in 1921 that reticulocytes tend to be concentrated at the top of the column of centrifuged red cells (8). Ultracentrifugation has been used to improve resolution (9). However, the rapid packing of red cells that occurs during the first few seconds prevents further migration of cells of different density. This problem is obviated by the use of buoyant density, where each cell is free to seek the position corresponding to its own specific gravity. A continuous gradient

\[
F_t = \frac{S_t}{S_0} = \frac{(1 - R)^t - (1 - R)^E}{1 - (1 - R)^E} + \frac{1 - (1 - R)^E}{A}
\]

(for days 0–50) \[1\]

\[
F_t = \frac{S_t}{S_0} = \frac{(1 - R)^E(1 - A)^{-E}}{1 - (1 - R)^E} + \frac{1 - (1 - R)^E}{A}
\]

(for day > 50) \[2\]
allows a progressive density-related distribution of red cells (10).
This technique, however, is suitable only for separation on a
minute scale, and considerable remixing may occur after cen-
trifugation. Discontinuous density gradients and separation of
the isolated layers by tube slicing result in density separation
of a degree comparable to that of continuous gradients, with
high reproducibility. This technique was originally developed
with solutions of crystalline bovine serum albumin, which are
both expensive and cumbersome to prepare (2). The separation
technique has recently been simplified by the introduction of
arabino-galactane as a supporting medium (4). The inherent
advantages of this medium make it conceivable to transfer the
technique of buoyant density gradient centrifugation from the
analytical laboratory to the blood bank.

In early studies using $^{59}$Fe as a label, it was suggested that the
separation of red cells by density resulted simply in enrichment
of the top fractions with the youngest red cells (11). Since $^{59}$Fe
is rapidly reutilized, there is a rapid diffusion of the label
throughout the red cell column, making evaluation of the
fractionation efficiency impossible after the first few days.
Labeling in vitro with [2-14C]glycine of a cohort of cells of very
narrow age span has demonstrated a time-dependent pro-
gressive movement of the radioactive label from the top to the
bottom layer. Thus, the increase in density with cell age is not
limited to the transition between reticulocyte and mature red
cell, but is a phenomenon which continues progressively
throughout the red cell life span (2). The experiments reported
in the present study offer additional evidence of the continuous
relationship between red cell age and cell density.

The relationship between cell age and position in the density
gradient can be predicted with precision for human red cells,
taking advantage of their symmetrical life-span curve and lack of
random loss. It is possible to develop a mathematical model
that translates in terms of in ovo life span the changes in age-
dependent properties of the red cells observed in fractions of
different densities. This technique has provided information
not only on the biochemical mechanism of red cell aging, but
also on the nature of certain genetically determined red cell
defects (12-14).

The present study was directed at evaluating whether it is
possible to use buoyant density separation to prepare a sizeable
red cell fraction with improved survival, suitable for transfusion
into humans. For this purpose, the density gradient must be
simplified and reduced to a single solution. The results obtained
indicate that, at least in rabbits, the resolution achieved meets
the theoretical expectation. Preliminary experiments indicate
that it is possible to separate human blood in a plastic transfusion
bag on a single layer of arabino-galactane. However, at the
present time, the application of this technique to human use
must await completion of the necessary tests of toxicity and
antigenicity and Food and Drug Administration approval for
pilot studies. The theoretical premises and the experimental
data in animals appear quite promising. Arabino-galactane is
used as a food additive (15) and appears to have negligible
toxicity in animals. Despite the fact that arabino-galactane does
not adhere to the red cell membrane, a minute amount will
always remain in the supernatant of the fractionated red cells,
even after multiple washes. For this reason, it appears essential
to establish its lack of antigenicity more definitely.

These studies indicate that it may be possible to prepare red
cells with improved survival, for transfusion to patients with
chronic anemias for whom reduction of the iron overload is of
critical importance. The promising technique described in this
study could easily be adapted to human use, with blood col-
lected through the ordinary blood bank routine. The fraction-
ation could be performed by all blood banks or by centralized
services which could keep the fractionated cells in the frozen
state. The advantage to the patient in need of chronic transfu-
sion will be not only one of spacing the transfusion in time by
a factor of 1.5, but also of avoiding the unnecessary transfusion
of the oldest red cells, which contribute equally to the iron
overload as do younger red cells but offer only minimal phys-
iological advantage to the patients. Should arabino-galactane
itself prove to be unsuitable for clinical use, it may be possible
to explore other media or alternative methods of density sep-
aration of red cells, such as the principle of the "elutriator"
centrifuge (16).

Transfusion of younger red cells with improved survival
prepared by using cells separated by density appears to be a new
approach to the transfusion therapy of patients with chronic
anemia. This, in combination with the recent advances in iron
chelation therapy (17, 18), may result in significant prolongation
of life, especially in patients with thalassemia major.

Appendix

A constant number of red cells ($a_0$) is formed by the rabbit each
day. The total number of red cells present at time 0 ($S_0$) rep-
resents the sum of the number of red cells formed that day and
of the surviving cells formed on previous days, affected in the
rabbit by both random (R) and aging (A) loss.

When the fate of an aliquot of red cells labeled with $^{51}$Cr at
time 0 ($S_0$) is considered on any subsequent day ($t$), the fraction
remaining ($F_t$) is given by the ratio of surviving labeled cells
($S_t$) relative to the number of originally labeled cells ($S_0$): ($F_t
= S_t/S_0$). The life span of rabbit red cells exhibits a biphasic rate
of decline. Initially, an exponential rate of decline, due to
random loss, affects red cell survival, until a time ($E$) when the
finite life span is reached and an accelerated rate of decline, due
to aging loss, becomes preponderant.

At any time $t$, up to $E$ days, the total sum consists of all cells
of age $\leq E$ as well as of all cells of age $>E$; after $E$ days, only
cells of age $>E$ remain. For $t = 0, 1, \ldots, E$:

$$S_t = \sum_{i=t}^{E} a_0(1 - R)^i + \sum_{j=1}^{\infty} a_0(1 - R)^E(1 - A)^j$$

[3]

Making the summation, and recognizing that when $j$ ap-
proaches $\infty$ the term $(1 - A)^j$ becomes negligible, a satisfac-
tory approximation is:

$$S_t = a_0 \left[ \frac{(1 - R)^t - (1 - R)^E}{R} \right] + \frac{(1 - R)^E}{A}$$

[4]

The number of cells on day 0 is:

$$S_0 = a_0 \left[ \frac{1 - (1 - R)^E}{R} \right] + \frac{(1 - R)^E}{A}$$

[5]

Hence, the fraction of cells remaining for days 0 to $E$ is equal
to (Eq. 4)/(Eq. 5) for $t = E + 1, \ldots, \infty$:

$$S_t = \sum_{j=t}^{\infty} a_0(1 - R)^E(1 - A)^j$$

[6]

By summing and simplifying, this becomes:

$$S_t = a_0 \left[ \frac{(1 - R)^E(1 - A)^{t-E}}{A} \right]$$

[7]

Hence, the fraction of cells remaining for days $E + 1$ to $\infty$ is
equal to (Eq. 7)/(Eq. 5).

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