Biotinylated Erythrocytes: In Vivo Survival and In Vitro Recovery

By Takashige Suzuki and George L. Dale

Rabbit erythrocytes were biotinylated by reaction with N-hydroxysuccinimido-biotin; the average level of biotinyl-
tation was 25,000 molecules per erythrocyte. These biotin-
lylated cells exhibited a normal survival rate when reinfused into
rabbits. Two studies demonstrated that the biotin label was
stable in vivo. The first was a double-labeling experiment where the biotinylated erythrocytes were also
labeled with ^14C-cyanate; on reinfusion, the loss of biotinyl-
ated erythrocytes and ^14C-cyanate label occurred in uni-
son. The second study demonstrated that biotinylated
erythrocytes that had been reinfused into rabbits could
later be selectively isolated by attachment to an avidin
support. This technique will facilitate a variety of studies
that require the ability to label a specific cohort of cells in
vitro and then reisolate those same cells after in vivo
recirculation.

THE MECHANISMS that govern erythrocyte senes-
cence have been extensively studied, and numerous
hypotheses have been proposed; however, definitive studies
have been hindered by the lack of techniques that allow
isolation of well-defined age groups of erythrocytes. Gener-
ally the isolation of specific erythrocyte subpopulations has
relied on physical techniques based on putative changes in
aged erythrocytes such as density or volume alterations.
However, the ability of existing physical techniques to give
unequivocal results when trying to isolate a defined age
group of erythrocytes has been questioned. A number of other procedures for the isolation of aged
erthrocytes have been reported. One interesting technique
allows the isolation of senescent rat erythrocytes. In
this procedure, a large number of starting animals are used.
At frequent intervals, a fraction of the group is killed to provide
to hypertransfuse the remaining rats. The steadily
decreasing number of surviving rats will not synthesize new
erthrocytes while they are polycythemic from the transfu-
sions, resulting in a few animals at the end of the experiment
having "aged erythrocytes." While this technique is quite
effective, it is also labor-intensive and consumptive of experi-
mental animals. Another procedure that is very preliminary
would isolate aged erythrocytes on the basis of surface
immunoglobulin levels, with the assumption that aged
erthrocytes have increased levels of bound immunoglobu-
lin.

Another relevant technique is found in the work of Allison
and Burn. These investigators transfused blood group O
erthrocytes into normal blood group A volunteers; the
mismatched cells were then selectively recovered by aggluti-
nation of the A erythrocytes. The technique allowed the
isolation of erythrocytes up to 102 days after transfusion, but
the utility of the procedure was hindered by the limitations of the
differential agglutination step.

In this report, we describe a novel approach to the isolation of
specific subpopulations of rabbit erythrocytes. This is
achieved by the biotinylation of erythrocytes, demonstration that these biotinylated cells have a normal in vivo survival in
the rabbit, and specific in vitro recovery of the biotinylated
erythrocytes. This technique, therefore, allows the specific
tagging of erythrocytes with a biocompatible label along with
the ability to recover this labeled subpopulation after reinfu-
sion into a rabbit.

MATERIALS AND METHODS
Polystyrene beads (200 to 400 mesh, 12% cross-linked, No. 4023)
were purchased from Polysciences, Inc, Warrington, PA. Avidin,
biotin, collagenase (type IV, 360 U/mg), bovine serum albumin
(BSA), 2-(4'-hydroxyazobenzene)-benzoic acid (HABA), and suc-
cinic anhydride were obtained from Sigma Chemical Co, St Louis.
N-Hydroxysuccinimido-biotin (NHS-biotin) was from Pierce
Chemical Co, Rockford, IL, and gelatin was from Difco Laborato-
ries, Detroit. N,N-Dimethyl-formamide (DMF) was purchased
from Eastman Kodak Co, Rochester, NY. ^14C-potassium cyanate
was obtained from Amersham, Arlington Heights, IL. All other
chemicals were of reagent grade or better.

Synthesis of Reagents
Succinylated BSA was synthesized by the method of Chu et al
with slight modifications. One gram of BSA was dissolved in 100 mL
of 0.5 M NaHCO3, pH 8.0. Succinic anhydride, 800 mg, was added
as 200-mg aliquots to the BSA solution over 60 minutes at room
temperature, and the pH maintained at 8.0 with NaOH. The
product was dialyzed against three changes of 2 L of 10 mmol/L
sodium phosphate, 150 mmol/L NaCl, pH 7.5 (PBS) at 4°C.
Succinylated BSA was stored at a protein concentration of 8.3
mg/mL or 120 μmol/L.

Biotinylated gelatin was synthesized by the following procedure:
Gelatin (60 mg) was dissolved in 10 mL of 50 mmol/L sodium
borate, pH 8.0, with mild heating. After cooling, NHS-biotin (4 mg
in 100 μL DMF) was added, and the mixture was incubated at room
temperature for 60 minutes. The biotinylated gelatin solution (Bio-
tin-Gelatin) was dialyzed against two changes of 1 L of PBS for four
hours at room temperature, and the solution was stored at 20°C.

Spectrophotometric determination of biotin bound to gelatin:
Biotin molecules bound to gelatin were determined by the method of
Green. The HABA dye binds weakly to avidin with a shift in the
dye's spectral properties; biotin easily displaces the HABA with a
concomitant change in the visible spectrum. An avidin–HABA
complex consisting of 0.2 mg avidin/mL of 0.25 mmol/L HABA,
0.1 mol/L sodium phosphate buffer, pH 7.0, was prepared. Different
amounts of biotinylated gelatin (10 to 120 μg) were added to 2 mL of
avidin–HABA complex, and their optical densities were measured at
500 nm. The biotin concentration was calculated as reported
and found to average 94 nmol biotin/mg gelatin.

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**Biotinylation of Erythrocytes**

Twenty milliliters of blood obtained from a male rabbit (New Zealand White, 2 to 3 kg) by auris arteriopuncture was collected into a heparinized tube. The blood was washed in PBS three times, and the buffy coat was removed. The cells were suspended to a 10% hematocrit in PBS containing 15 mmol/L glucose. Ten milliliters of suspension was added to 15 μCi [14C]-potassium cyanate, 0.83 μCi/μmol, and the mixture was incubated at 37°C for 30 minutes in a shaking bath. Succinylated BSA, 295 nmol, was then added to the cell suspension and gently shaken for three minutes at room temperature. Finally, 295 nmol NHS-biotin (100 μg, stock solution 2 mg/mL of DMF) was added to the mixture, and the incubation was continued at 37°C for 60 minutes. The mean substitution level with this recipe was 26,600 ± 7,300 (n = 6) biotin molecules per erythrocyte.

The [14C]-labeled and biotinylated erythrocytes were washed three times in PBS and resuspended to a 50% hematocrit. To examine cell shape, a portion of the cells was fixed in 1% glutaraldehyde in PBS; the absence of abnormal cells (echinocytes and elliptocytes) was confirmed by light microscopy. The biotin and [14C] content of the cells was determined as described below. The remaining erythrocyte suspension was reinfused by auris venipuncture into the same rabbit from which the original blood was obtained. At five minutes after reinfusion, 2 mL of blood was obtained as a T = 0 sample by auris arteriopuncture of the opposite ear used for reinfusion. Blood samples were taken at regular intervals for up to 60 days.

**Quantitation of [14C] and Biotin Content of Erythrocytes**

The collected blood was filtered through cellulose to remove leukocytes and platelets. Erythrocytes were washed three times with PBS and left at approximately 20% hematocrit; an accurate hematocrit value of the resuspended sample was determined with a micro hematocrit centrifuge. The [14C] radioactivity level was measured as follows: The erythrocyte suspension (usually 200 to 400 μL) was mixed with 1 mL of 10% sodium dodecylsulfate in a glass scintillation vial and shaken at 37°C. After 15 minutes, 65 μL of 30% hydrogen peroxide was added to bleach the hemoglobin-rich solution and the incubation continued at 37°C for 30 minutes. Finally, 50 μL of neat β-mercaptoethanol was added to neutralize any residual peroxide with a final incubation at 37°C for 30 minutes. After adding 10 mL of scintillation fluid (Safety Solve) to the colorless mixture, the samples were left at room temperature for at least two hours before counting to allow chemiluminescence to decay.

Biotin bound to erythrocytes was quantitated indirectly by saturation binding of 125I-avidin. Three and one-half milliliters of washed cell suspension (20% hematocrit) in PBS were added to a 25-mL Erlenmeyer flask along with 3 mL PBS containing 1 mg/mL BSA. Each flask received 50 μg of 125I-avidin (approximately 1,000 cpm/μg). A background level of nonspecific 125I-avidin binding to erythrocytes was determined by carrying out the incubation in the presence of 0.5 mmol/L biotin to overwhelm the small amount of erythrocyte–bound biotin. The mixture of erythrocytes and 125I-avidin was incubated with shaking at 37°C for 60 minutes. The cells were washed three times with PBS and cell–bound 125I radioactivity was determined.

The avidin concentration (A), in μg avidin/mL RBC, was calculated as follows:

\[ A = \frac{(B_1 - B_2)}{CR} \]

where \( B_1 \) is the radioactivity (cpm) of the sample without biotin, \( B_2 \) is the radioactivity of the sample with 0.5 mmol/L biotin, C is the specific radioactivity of the 125I-avidin (cpm/μg), and R is the packed erythrocyte volume present in the assay. The number of avidin molecules (N per erythrocyte), which is used as an estimate of biotin molecules on a cell, was calculated as follows:

\[ N = \frac{(A \times 10^8 \times 6.02 \times 10^{23})}{68,000 \times 10^9} \]

where the molecular weight of avidin is 68,000 g/mol, Avogadro's number is \( 6.02 \times 10^{23} \) molecules/mol, and the number of erythrocytes in 1 mL of packed cells is estimated as 10^9.

**In Vitro Recovery of Biotinylated Erythrocytes**

**Preparation of avidin-biotin gelatin coated beads.** Four milligrams of biotin–gelatin and 2 mL of PBS were added to 500–mg polystyrene beads in a polystyrene tube (17 x 100 mm, Falcon 2059) and mixed on a rotator for 60 minutes. The beads with bound biotin–gelatin (biotin–gelatin–beads) were washed five times with PBS by gentle centrifuging at 300 g for five seconds. Avidin (750 μg) and 2 mL PBS were added to the washed biotin–gelatin–beads and rotated at room temperature for 20 minutes. The beads were washed five times with PBS by gentle centrifuging at 300 g for five seconds. This final preparation is referred to as avidin–biotin–gelatin–beads.

**Recovery of biotinylated cells.** Biotinylated erythrocytes were withdrawn from rabbits, freed of leukocytes, and washed as detailed above. One milliliter of these erythrocytes (approximately 80% hematocrit) was mixed with 500 mg avidin–biotin–gelatin–beads in a 50-mL glass beaker and incubated on a rocking platform at room temperature for 60 minutes. The beads were transferred into a 15–mL plastic tube and washed with PBS by gentle centrifuging at 300 g for five seconds; the washing was repeated until the supernatant was clear.

To collect intact cells from the beads, collagenase (900 U in 10 mL PBS) was added to the beads with bound biotinylated erythrocytes in a 25–mL Erlenmeyer flask and the mixture incubated at 37°C for fifteen minutes. The mixture was centrifuged briefly twice at 300 g for five seconds to remove beads, and the supernatant containing biotinylated erythrocytes was taken.

**RESULTS**

Rabbit erythrocytes were reacted with NHS–biotin to tag the extracellular surface with covalently attached biotin. These biotinylated erythrocytes were then reinfused into normal rabbits and could be subsequently recovered by cell affinity chromatography on an avidin support. Figure 1 depicts the overall experimental scheme. Figure 2 shows the number of biotin molecules bound to the erythrocytes as a function of the NHS–biotin concentration. Early experiments indicated that the erythrocytes needed approximately 15,000 biotin molecules per cell if the cells were to be successfully recovered by attachment to an avidin support (data not shown). Therefore, erythrocytes with an average of 25,000 to 30,000 biotin molecules per cell were used for most experiments to ensure that the cells could be recovered in vitro, as outlined below.

Rabbit erythrocytes containing an average of either 13,400 or 27,000 biotin molecules per cell and labeled with [14C]-cyanate were reinfused into normal rabbits. Subsequently, Samples were taken from the rabbits at regular intervals for 93 days; the reported mean life span of rabbit erythrocytes is approximately 60 days. The data in Fig 3 demonstrate that the biotinylated and [14C]-labeled erythrocytes had a viability indistinguishable from that of control [14C]-labeled erythrocytes.
BIOTINYLATED ERYTHROCYTES

Fig 1. Overall scheme for labeling and recovery of rabbit erythrocytes. Erythrocytes were biotinylated (B) by reaction with NHS-biotin; these biotinylated cells could be selectively recovered by attachment to avidin (A) coated polystyrene beads.

Fig 2. Derivatization of erythrocytes with NHS-biotin. Rabbit erythrocytes in PBS were reacted with various concentrations of NHS-biotin. The number of biotin molecules bound per erythrocyte was determined by saturation binding of 125I-avidin to the cells (see Materials and Methods section for details).

Fig 3. In vivo survival of biotinylated and control erythrocytes. Blood was drawn from three rabbits; two of the preparations were biotinylated (open circles, 27,000 biotin molecules per cell; triangles, 13,400 biotin molecules per cell) and the third served as a control (closed circles). All three preparations were labeled with 14C-cyanate and reinfused into the respective donor rabbits. Samples were drawn at regular intervals and the 14C remaining expressed as percent of starting level (T = 5 minutes sample).

Figure 4 verifies that the biotin present on the erythrocytes is stable in vivo. For these experiments it was necessary to biotinylate the erythrocytes more heavily (126,000 biotin molecules per cell) in order to measure the actual level of erythrocyte-bound biotin after reinfusion; this increased level of biotinylation results in a moderately decreased in vivo life span for these cells. As shown in Fig 4, the level of erythrocyte-associate 14C-cyanate and biotin present in the rabbits decreased in parallel, indicating that the biotin is stable on the erythrocytes in vivo. This stability of the biotin tag is particularly significant in that serum contains biotinidase, an enzyme capable of cleaving biotin from some peptide substrates. While biotinidase is reported to be active only with low molecular weight substrates, the possi-
ability that prolonged exposure of biotinylated erythrocytes to biotinidase might result in the enzymatic removal of some biotin could not be excluded a priori.

The in vitro recovery of biotinylated erythrocytes was first examined using synthetic mixtures of 95% control and 5% biotinylated erythrocytes to mimic the approximate ratio expected in later experiments. The biotinylated erythrocytes were also labeled with $^{51}$Cr–sodium chromate, which served as a marker for the efficiency and accuracy of the in vitro recovery system. Table 1 shows the data from a representative experiment. The efficiency of recovery of biotinylated erythrocytes varied from 25% to 50% using the conditions described in the Materials and Methods section. More important than the efficiency of recovery is its accuracy, ie, how much contamination is there by nonbiotinylated erythrocytes? This was examined by determining the ratio of radioactivity to hemoglobin (Hb) in the recovered cells. If nonbiotinylated and nonradioactive control cells are bound to the avidin–biotin–gelatin–beads, the cpm/Hb ratio would be dramatically altered. As shown in Table 1, the recovered cells have a cpm/Hb ratio that is approximately 90% of the specific activity of the pure, biotinylated erythrocyte population.

The recovery of biotinylated erythrocytes after reinfusion into rabbits is shown in Table 2. As discussed above, the specific recovery of $^{14}$C-labeled and biotinylated erythrocytes is indicated by the constant $^{14}$C/Hb ratio over time. Also shown are the $^{14}$C/Hb ratios for the unfractionated samples before attachment to the avidin–biotin–gelatin–beads. As expected, this latter ratio drops as the biotinylated cells are progressively removed from circulation; however, the $^{14}$C/Hb ratios for the recovered cells remain constant, again indicating the selectivity of the in vitro recovery.

Intact, biotinylated erythrocytes could be recovered from the avidin–biotin–gelatin–beads by incubation with collagenase; the collagenase enzymatically degrades the gelatin bound to the polystyrene beads, thereby disrupting the anchor that holds the biotinylated erythrocytes to the beads. The yield of intact cells varied between 33% and 66% of the

| Table 1. Model Studies on Recovery of Biotinylated Erythrocytes |
|------------------|------------------|------------------|
| Sample          | Bound to Beads* (%) | Recovered After Collagenase Treatment of Erythrocytes/Beads* | Specific Activity of Recovered Cells (cpm/mg Hb) | Control (%) |
| A               | 32.4             | 16.0            | 1605             | 91.1  |
| B               | 29.0             | 14.5            | 1595             | 90.5  |
| C               | 31.0             | 16.2            | 1520             | 86.2  |

A synthetic mixture of 95% control erythrocytes and 5% biotinylated and $^{51}$Cr–labeled erythrocytes was mixed with avidin–biotin–gelatin–beads as described in Materials and Methods.

*Recovery is percent of total radioactivity of biotinylated erythrocytes before attachment to the avidin–biotin–gelatin–beads.

†Specific activity of starting biotinylated erythrocyte population was 1760 cpm/mg Hb, and the specific activity of the synthetic mixture of 95% control and 5% biotinylated, $^{51}$Cr–labeled erythrocytes was 75.3 cpm/mg Hb.

bound cells; this large variability is not understood and is currently under investigation.

**DISCUSSION**

This report describes a technique for the biotinylation of rabbit erythrocytes that does not affect the in vivo survival of the derivatized cells but still allows for the specific isolation of the biotinylated erythrocytes by an avidin support. Figure 1 shows the general experimental scheme.

The level of biotinylation is quite low, with an average of 26,600 biotin molecules per erythrocyte. However, preliminary experiments revealed that the erythrocyte morphology was altered by the NHS–biotin incubation. Greater than 90% of the cells after the derivatization were echinocytes rather than normal biconcave discs, although an almost normal shape could be restored to the cells by repeated washings in the presence of BSA. We subsequently found that inclusion of succinylated BSA, equimolar to that of NHS–biotin, prevented the shape change and resulted in a cell morphology for the biotinylated erythrocytes that was indistinguishable from the control cells. The succinylated BSA serves as a nonnucleophilic carrier for the hydrophobic NHS–biotin. In the presence of this carrier, the NHS–biotin is presumably prevented from intercalating into the erythrocyte membrane and causing a reversible shape change similar to that observed with several lipophilic drugs.

As detailed in the Results section, the biotinylated erythrocytes had an in vivo survival indistinguishable from that of control cells. One initial concern with this technique was that the rabbit might produce an immune response against the biotinylated cells. Clearly the data in Fig 3 would suggest that this is not a major concern, since antibody produced during the 53 days that biotinylated erythrocytes were present would be expected to affect the cell’s survival. However, additional experiments are in progress to systematically examine the question of whether an immune response occurs.

In vitro recovery of the biotinylated erythrocytes was achieved with an avidin–biotin–gelatin–bead support. The major advantage of this support is that the gelatin anchor can be cleaved enzymatically with collagenase, thereby releasing...
intact erythrocytes from the beads. It should be mentioned that the released cells still have bound avidin as well as biotin–gelatin fragments, which could potentially interfere with some analyses.

The impetus to develop this technique was an interest in erythrocyte senescence, which requires the isolation of aged erythrocytes. The utility of the procedure is shown by the following example. If a random population of rabbit erythrocytes is biotinylated and reinfused, then 50 days later the only surviving biotinylated cells will now be between 50 and 60 days old and represent those cells that were from zero to ten days old at the time of biotinylation. This procedure will, therefore, greatly facilitate the examination of putative factors related to erythrocyte senescence. However, if the biotinylated cells are isolated 30 days after reinfusion, the biotinylated population represents cells from 30 to 60 days old, a much broader range. An additional improvement in the technique would be to initially isolate reticulocytes for biotinylation. This would allow the subsequent recovery of a narrowly defined age group of cells at any time after reinfusion.

Another potential use for this biotinylation method would be as an alternative to the currently used radioactive chromium techniques for erythrocyte survival studies. The benefits would include not exposing the test subject to radioactivity but would be partially offset by the increased handling of the cells required for biotinylation. As outlined above, before this technique can be used in humans further experimentation will be required to determine if there is an immune response to the biotinylated cells.

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Biotinylated erythrocytes: in vivo survival and in vitro recovery

T Suzuki and GL Dale