Ann Hematol (1993) 67:81–87

Annals of
Hematology

Original article

Biotin labeling as an alternative nonradioactive approach to determination of red cell survival

G. Hoffmann-Fezer[1], J. Mysliwietz[1], W. Mörtlbauer[2], H. J. Zeitler[3], E. Eberle[2], U. Hönle[1], and S. Thierfelder[1]

[1] Institut für Immunologie, GSF, Marchioninistrasse 25, D-81377 München, Germany
[2] Institut für Medizinische Informatik und Systemforschung, GSF, D-85758 Neuherberg, Germany
[3] Institut für Klinische Hämatologie, GSF, D-81377 München, Germany

Received 25 September 1992 / Accepted 6 May 1993

Summary. Biotin labeling of red cells was studied using different approaches to see if biotinylation is a useful label for determination of erythrocyte survival. Mouse red cells were labeled with biotin, either in vivo by injection or in vitro. In vivo labeled red cells were followed up in some mice without transfusing the labeled erythrocytes. Furthermore, in vivo labeled as well as in vitro labeled red cells were transfused into syngeneic mice. The biotin label allows an easy discrimination between labeled and unlabeled red cells during FACS analysis, and it is relatively stable for at least 50 days. All the three different approaches give similar results. Mean red cell life spans of in vivo or in vitro labeled red cells either transfused or followed up in vivo were between 44 and 52 days (T_{50} mean 23.9 days) when red cell destruction was assumed to be only a result of senescence. Mean red cell life spans were between 8 and 18 days (T_{50} mean 9.5 days) when a random destruction independent of red cell age was suggested. All the survival slopes are neither simple linear functions of time nor logarithmic functions, but they show an overlay of linear function by a logarithmic function where the components of both are unknown.

Key words: Red cell survival – Biotin labeling

Introduction

Determination of red cell life span is currently performed by random labeling methods using one of the radioactive tracers: ^{51}Cr, $DF^{32}P$, or $DFP\text{-}H^{3}$ (diisopropylphosphorofluoridate). All of these tracers have disadvantages which make them less than ideal [15]. The oldest nonradioactive technique is the differential agglutination method of Ashby [1]. This method, as well as a more recently developed modification using an enzyme-linked antiglobulin test [10], suffers from the necessity of the use of transfused red blood cells incompatible with the blood of the recipient. Therefore, the recently reported random labeling technique using the fluorescent lipophilic probe PKH-3 [15] indicated considerable progress in labeling red cells.

We present here another nonradioactive label for life span studies – biotin – which allows good and stable random labeling in vivo and in vitro, as already shown using avidin-coated microbeads [7, 17]. Analysis of labeled cells can then be performed by flow cytometry much more easily than in a previous report, in which in vivo labeled red cells were evaluated by microscopic enumeration [8], or by binding to avidin-coated polystyrene beads [17].

Materials and methods

Animals

Female 10- to 12-week-old CBA/J mice originally obtained from the Jackson Laboratories (Bar Harbor, ME) were raised and maintained in our breeding facilities. They were fed with Altromin 1314 and 1324 (free of egg-white avidin).

Biotin labeling of erythrocytes

Two biotinhydroxysuccinimide esters were used. Biotin-X-N-hydroxy-succinimide ester (B-X-NHS, Calbiochem, San Diego/CA) was a commercial product. Caproylamidobiotin-*N*-hydroxysuccinimide ester (C-BNHS) was synthesized in our laboratories; the synthesis is described elsewhere [8]. The melting point of C-BNHS was higher (167°–169° C) than that of the purchased compound B-X-NHS (156°–163° C). This indicates that the C-BNHS is of higher purity and is therefore more efficient. In vivo labeling was performed with three daily doses of 0.75–1 mg of C-BNHS or B-X-NHS dissolved in 27–38 µl dimethylformamide and 250 µl PBS, injected i.v. on 3 consecutive days.

For in vitro labeling 1.35 mg B-X-NHS dissolved in 50 µl DMF and 4 ml PBS was added to 800 µl native blood containing heparin novo as anticoagulant and kept at 37° C for 30 min with gentle shaking. Red cells were washed with phosphate-buffered solution (PBS) and adjusted to 5×10^9 red cells/ml.

Correspondence to: G. Hoffmann-Fezer

Transfusion of biotin-labeled red cells

In vivo labeled red cells were removed by cardiac puncture. After either in vivo labeling or in vitro labeling, approximately 1×10^9 biotin-labeled red cells were injected intravenously in 300 µl PBS into syngeneic recipients.

Recovery of biotin-labeled red cells

Biotin-labeled red cells were followed up (a) after in vivo biotinylation without transfusion, (b) in mice transfused with in vivo biotin-labeled red cells, and (c) in mice transfused with in vitro biotin-labeled red cells. Short-term post transfusion tests were performed to evaluate a possible early destruction of transfused red cells 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 1 day, and 2 days after transfusion. Long-term follow-up was done 120 min, 1 day, and then every third or fourth day up to days 50–60. About 3 µl of blood (about 0.25% of total blood volume) was withdrawn from tail veins using a heparinized hematocrit capillary. Blood was diluted in PBS, and 6×10^6 red cells were incubated with an appropriate dilution of FITC-labeled avidin (Jackson Immuno-Research, Bar Harbor/ME) for 10 min at 4° C. Cells were not washed, because it was found that washing with PBS did not influence the results. Untreated syngeneic mouse red cells were used as negative controls. Red cells were analyzed by automated flow cytometry.

Flow cytometric analysis

Red blood cell samples were analyzed using a FACScan flow cytometer (Becton-Dickinson, Heidelberg) with an argon laser, 488 nm wavelength. Microbead standards (QFMS, Becton-Dickinson, Heidelberg) were used to ensure stable setting of the instrument throughout the analysis. Gates were set, using forward-angle light scatter and right-angle light scatter, to exclude analysis of aggregated cells or debris.

Fluorescence signals of 10000–15000 red blood cells were recorded as 1030 channel histograms using four orders of log or linear amplifiers. Control histograms were obtained by replacing the biotinylated experimental samples with untreated syngeneic erythrocytes. The fluorescence signals of the control were subtracted from experimental values of the transfused mice. The percentage of biotinylated cells and the mean fluorescence intensity were determined using FACScan software.

Statistics

Two regression models were fitted for each mouse. In model 1 we assume that all the red cells have the same lifespan L and cell destruction occurs only as a result of senescence. The probability $\varphi(a)$ that a cell will survive to a given age 'a' will be 1 if that age is numerically less than L, and 0 if the age is numerically greater than L. The survival function thus becomes $f(t) = 1 - t/L$; i.e., we get a linear relationship between the percentage of labeled red cells and time. In model 2 we assume that red cell destruction occurs as a result of random processes acting independently of cell age. The probability that a cell will survive to an age 'a' will be a decreasing function of time; thus $\varphi(a) = \exp(-a/L)$. The survival function is $f(t) = \exp(-t/L)$; i.e., we get a linear relationship between the logarithm of the percentage of labeled red cells and time. The parameter L is called mean cell lifespan and equals the time corresponding to 37% ($\exp(-1)$ exactly) survival [3]. In both models the parameter L was fitted by regression analysis techniques.

Additionally, the mean T_{50} biotin, i.e., the time corresponding to 50% survival, and the coefficient of determination r^2 was computed in each case. The latter statistic provides a measure of the success of the regression fitting in explaining the variability in the response variable. The value of r^2 is between 0 and 1, and the greater the value, the better the regression fits.

Results

Detection of biotin label in vitro

Biotin-labeled red cells were easily detected when stained with FITC-labeled avidin. In vivo biotinylation with C-BNHS resulted in stronger erythrocyte labeling than with B-X-NHS; this was shown by higher fluorescence intensity of labeled red cells. Nevertheless, B-X-NHS-labeled cells were clearly distinguishable from unlabeled red cells. A single intravenous injection of biotin compounds labeled the red cells too weakly for a clear discrimination between unlabeled and labeled red cells, but two, and especially three, injections produced an excellent signal-to-noise ratio (Fig. 1) on 100% of erythrocytes.

The fluorescence intensity of labeled red cells was reduced continuously from 100% on day 0 to 86.4% ± 3.2% on day 36 in mice whose red cells were labeled in vivo. In vitro labeled red cells showed a decrease in fluorescence intensity to 67.3% ± 7.3% on day 36 after transfusion. Nevertheless, labeled and unlabeled red cells were easily discriminated when fluorescence intensity was measured using linear amplification (Fig. 2). Negative controls with unbiotinylated red cells usually resulted in less than 0.1%, rarely up to 0.2%, unspecifically stained particles of red cell size; their variation is between 0.05 ± 0.03 and 0.17 ± 0.08 in different experiments.

Long-term studies were performed in different ways. At first, we followed up the life spans of in vivo biotin-labeled red cells. We expected to obtain the real life span, not influenced by possible damage to the red cells, by in vitro handling during the labeling procedure.

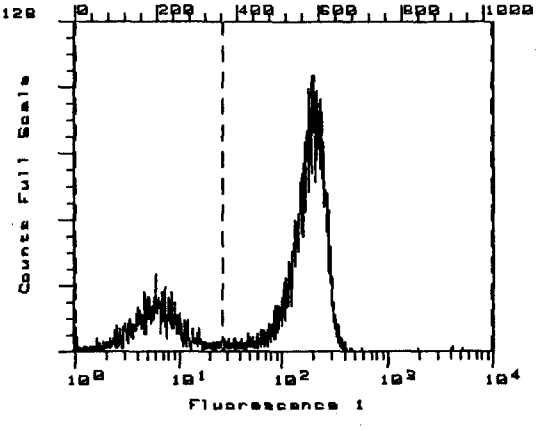

Fig. 1. FACS profile of red blood cells in vivo labeled with C-BNHS 9 days before shows a clear discrimination between labeled (*right*) and unlabeled, newly produced red cells (*left*)

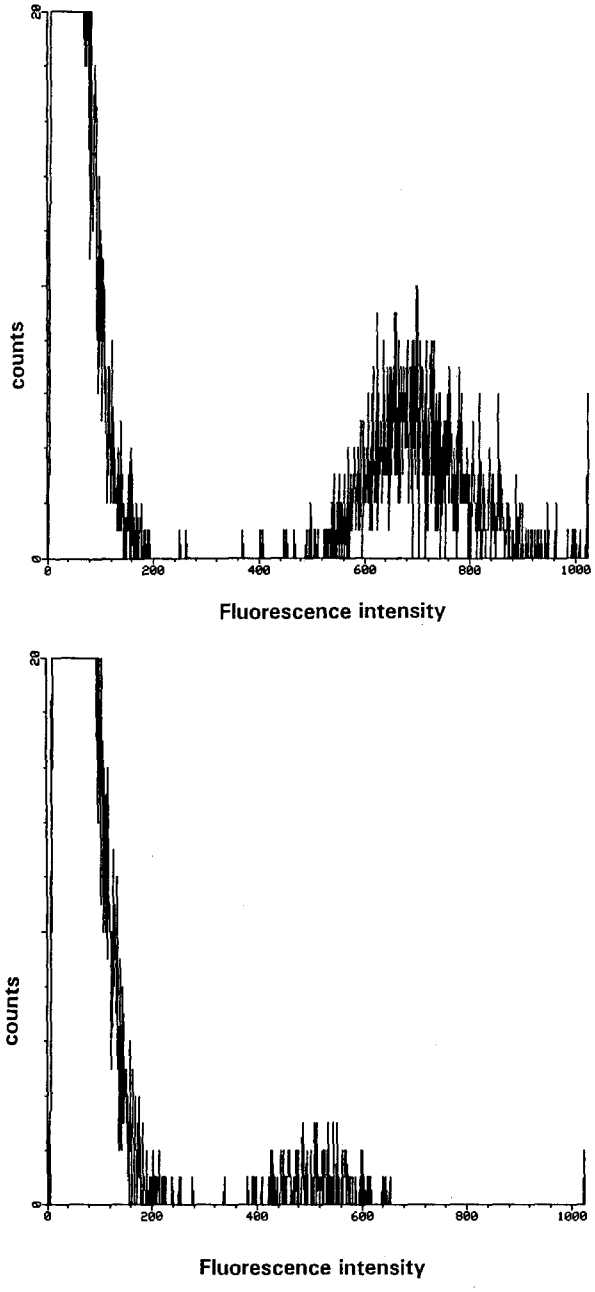

Fig. 2a, b. In vitro biotin-labeled and transfused red cells (**a**) 4 days before (**b**) 32 days before can be easily distinguished when fluorescence intensity is measured using linear amplification

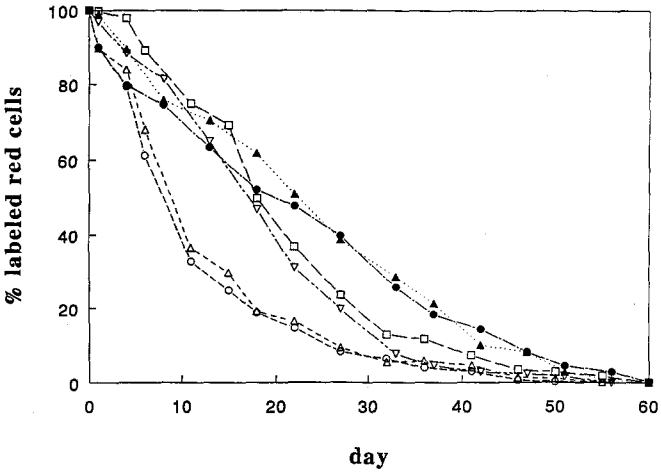

Fig. 3. Life span of in vivo labeled red cells following injection of three daily doses of biotin (each *symbol* represents one animal)

Table 1. Life spans of in vivo biotin-labeled red cells

	Model 1 (linear)			Model 2 (log)		
Mouse	L	T_{50}	r^2	L	T_{50}	r^2
1	45.7	22.8	0.9578	11.5	8.0	0.9404
2	51.1	25.6	0.9688	17.5	12.1	0.9692
3	47.0	23.5	0.9293	12.2	8.5	0.9638
4	44.9	22.4	0.8503	8.5	5.9	0.9921

L, Cell life span in model 1, mean cell life span in model 2; T_{50}, time corresponding to 50% labeled red cells; r^2, measure of the success of the regression

Life span of in vivo labeled red cells

When mice injected with three doses of biotin were bled over a 50-day period, labeled red cells were easily detected and decreased from 100% labeled red cells 2 h after the third i.v. injection to values below 1% on day 46 (Fig. 3). In contrast, from day 1 on after the last biotin injection, unlabeled, newly produced red cells appeared and increased with time, to nearly 100% on day 46.

Regression analysis showed T_{50} survival times between 22.8 and 25.6 days (mean 23.6 days) when model 1 (linear) was assumed, and between 8 and 12.1 days (mean 8.6 days) when model 2 (logarithmic) was fitted (Table 1).

Survival of transfused in vivo biotin-labeled red cells

Once information was obtained indicating that in vivo biotin labeling allowed determination of red cell survival, we wondered how long in vivo biotinylated red cells would survive after transfusion into syngeneic recipient mice, particularly in comparison with the life span of red cells in in vivo labeled, nontransfused mice. We transfused 1.1×10^9 biotinylated red cells. Female CBA mice contain about 12.5×10^9 red cells [2, 16], so we expected to find about 8% labeled erythrocytes in the blood samples drawn on day 1. Multiple blood samples were taken during the first day, 5, 15, and 30 min and 1, 2, 3, and 24 h after intravenous infusion of biotin-labeled red cells for short-term post-transfusion studies. Although the number of biotin-labeled cells was in the expected range of about 8% labeled red cells already 5 min after transfusion, values increased slightly up to the highest values at 2 or 3 h after injection of labeled cells (Fig. 4).

These short-term post-transfusion studies showed that the percentages of labeled red cells were not sta-

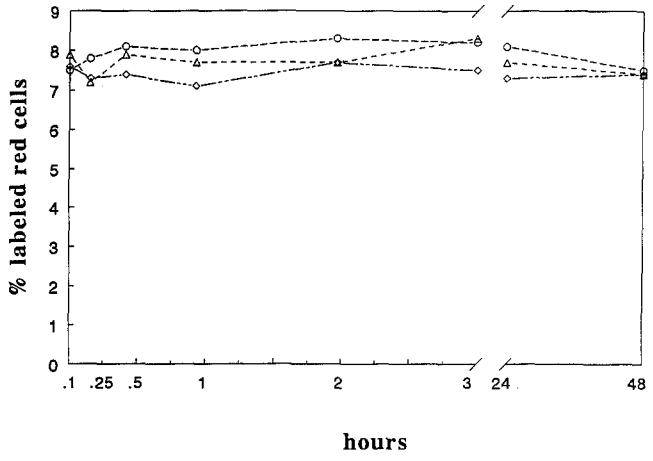

Fig. 4. Short-term post transfusion survival of in vivo biotin-labeled and transfused red blood cells shows highest percentages 2 or 3 h after transfusion (each *symbol* represents one animal)

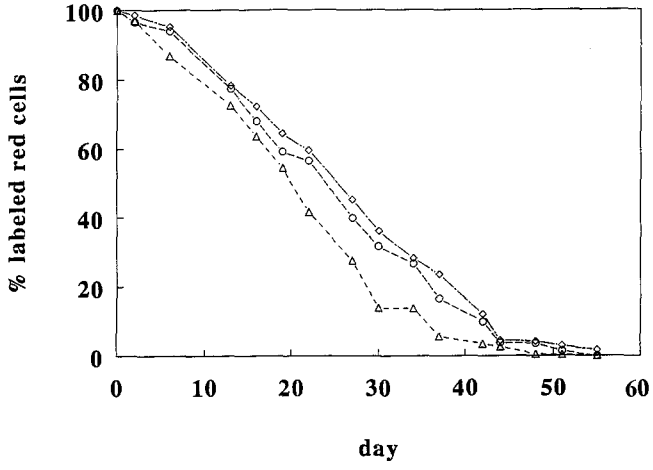

Fig. 5. Long-term survival of in vivo biotin-labeled and transfused red blood cells (each *symbol* represents one animal)

ble before 2 or 3 h after transfusion. Two-hour post-transfusion values were, therefore, set as the 100% reference in the following experiments.

Thereafter, other mice were bled over a 55-day period. As shown in Fig. 5, labeled cells disappeared steadily and reached values below 5% on day 42.

Regression analysis calculated T_{50} survival times between 22.6 and 25.3 days (mean 24.1 days) of in vivo labeled and transfused red cells when model 1 (linear) was assumed and T_{50} survival times between 8.4 and 12.4 days (mean 10.9 days) when model 2 (logarithmic) was fitted (Table 2).

Survival of in vitro biotin-labeled red cells

Finally, red cells were biotin-labeled in vitro with 1.7×10^{-10} mg B-X-NHS per erythrocyte including plasma proteins. This amount is in the same range as the injected total amount of 2.25–3 mg, which corresponds to about 2.4×10^{-10} mg per erythrocyte (disregarding the plasma proteins and endothelial cells of the vascular system). Our in vitro biotin-labeled red cells do not show signs of echinocytosis (as described by Suzuki et al. [17] for washed biotin-labeled red cells). These in vitro biotin-labeled cells were injected into mice and followed up for a 61-day period. Biotin-labeled red cells decreased slowly to below 7% on average on day 42 (Fig. 6).

Table 2. Survival times of in vivo biotin-labeled, transfused red cells

	Model 1 (linear)			Model 2 (log)		
Mouse	L	T_{50}	r^2	L	T_{50}	r^2
1	50.6	25.3	0.9941	17.9	12.4	0.9078
2	45.2	22.6	0.9799	12.2	8.4	0:9061
3	48.9	24.4	0.9933	17.1	11.8	0.8990

L, Cell life span in model 1, mean cell life span in model 2; T_{50}, time corresponding to 50% labeled red cells; r^2, measure of the success of the regression

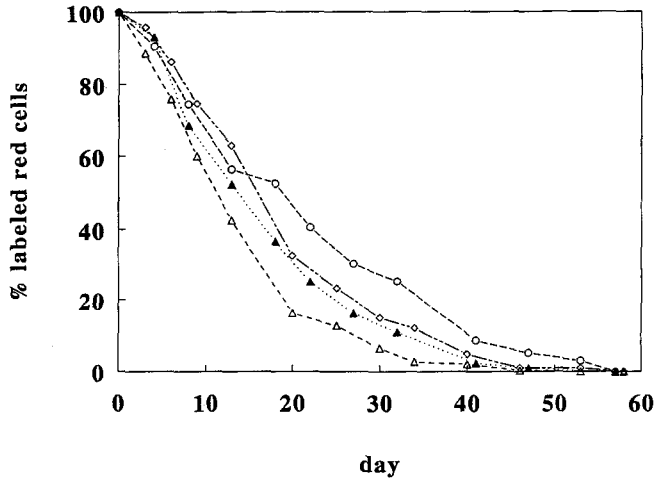

Fig. 6. Survival of in vitro biotin-labeled and transfused red blood cells (each *symbol* represents one animal)

Table 3. Survival times of in vitro biotin-labeled transfused red cells

	Model 1 (linear)			Model 2 (log)		
Mouse	L	T_{50}	r^2	L	T_{50}	r^2
1	48.5	24.2	0.9657	14.7	10.2	0.9737
2	44.2	22.1	0.9020	10.9	7.6	0.9814
3	43.7	21.8	0.8926	9.9	6.9	0.9830
4	52.1	26.0	0.9868	14.9	10.3	0.8918
5	51.7	25.8	0.9782	15.9	11.0	0.8865
6	47.5	23.8	0.9572	12.2	8.5	0.9689

L, Cell life span in model 1, mean cell life span in model 2; T_{50}, time corresponding to 50% labeled red cells; r^2, measure of the success of the regression

Regression analysis showed T_{50} survival times between 21.8 and 26.0 days (mean 24 days) when model 1 (linear) was used and between 6.9 and 11.0 days (mean 9.1 days) when model 2 (logarithmic) was assumed (Table 3).

Discussion

Suitability of biotin as labeling substance

The aim of this paper is to demonstrate the excellent suitability of biotin as a nonradioactive label for determination of red cell survival. Biotin labeling of rabbit erythrocytes was performed with B-X-NHS to isolate aged red cells [7, 17, 18]. Furthermore, biotin labeling has already been used successfully for measurement of red cell volume in adult patients [4] and in preterm infants [9].

Comparing the C-BNHS from our laboratory with the commercial product B-X-NHS, we achieved a more efficient cell labeling because of our improved chemical synthesis. In contrast to the melting point of the commercial B-X-NHS (156°–163° C; with decomp.), our C-BNHS exhibited a 10°-higher melting point, indicating that commercial products may still contain degradation or by-products.

As shown by our results, biotin fulfills all the requirements of an ideal labeling substance for random labeling procedures. The main advantage of biotin over PKH-3 [15] and other fluorescent labeling substances is that, being a vitamin, it is not toxic and it is a physiological component of organisms [14]. Unfortunately, DMF is necessary as a solvent for c-BNHS and B-X-NHS. A solution of the biotin esters in warm PBS labels red cells weaker than lymphoid cells (unpublished observation). The injection 30–40 mg of DMF were well tolerated. The LD_{50} of DMF is about 4 g/kg in a 20% solution [11]. In those experiments where labeled red cells were transfused, the majority of DMF was removed by washing the red cells (J. G. Filser, manuscript in preparation).

Labeling with biotin can be performed easily and is relatively stable for at least 50 days. Although some loss of labeling intensity occurs, as shown by a shift of the histogram to the left, especially after in vitro labeling, the detection of labeled red cells is not disturbed. Loss of labeling intensity is higher after in vitro labeling than after in vivo labeling; this may presumably be due to biotinidase, a biotin hydrolase enzyme [5]. Intravenous injection of biotin labels not only red cells but also plasma proteins and endothelial cells of the vascular system (unpublished observation). Therefore biotinidase can react with more substrate on other than red cells in the in vivo labeled mice than in animals with transfused labeled red cells. It follows that relatively less label of the in vivo labeled red cells disappears. A loss of membrane proteins during the aging of the red cells should induce a similar loss in both groups of labeled red cells. Theoretically, reutilization cannot be expected. A relabeling of the proteins of blood cells is impossible, because the strongly reactive group of the N-hydroxsuccinimide ester was reacted with a chemically nonreactive acylamide bond. Even after a cleavage of the bond (e.g., by the action of biotinidase) a reutilization of biotin for cell labeling has to be excluded because (a) the coupling procedure (biotinylation of proteins) is based upon an irreversible split-off of the N-hydroxysuccinimide moiety, and (b) mammalian cell systems have never been reported to form biochemically active forms of the vitamin biotin for cell labeling. Indeed, regarding our transfusion experiments, we found no signs of reutilization of the biotin label.

Detection of biotin-labeled red cells

A further advantage is that biotin is easily detectable by FITC-labeled avidin and easily analyzed by flow cytometry. This method requires just 3 µl of blood (even 1 µl would be sufficient). This is in contrast to former attempts by Cavill et al. [4] and Hudson et al. [9], who used NHS-LC-biotin as a red cell label and were not able to detect biotin-labeled red cells 7 days after injection; they described total clearing of labeled red cells in preterm infants after 3–4 weeks, although they used the technique of biotin-labeled red cells successfully for the measurement of total red cell volumes. A difference between their procedure and ours is that we labeled the red cells with the approximately 20-fold amount of B-X-NHS (not NHS-LC-biotin) but included plasma of unwashed red cells. Labeling of washed red cells induced lysis of red cells also in our hands. Suzuki et al. [17], Dale et al. [7], and Zimran et al. [18] used a time-consuming method for isolating biotinylated erythrocytes with avidin-coated polystyrene beads which is less suitable for quantifying labeled cells. We used FACS analysis of biotin-labeled red cells stained with FITC-labeled avidin. This method enabled us to analyze only red cells and to gate out other labeled cells. We can detect both intensity of labeling per individual red cell and number of labeled red cells. When we adapted the rare-event analysis method described by Cupp et al. [6] for the analytical procedure, i.e., linear measurement for a better distribution and subtraction of the values of negative controls, we were able to determine biotin-labeled red cell percentages below 1%. The results in different experiments were similar.

Advantages of in vivo labeling

From a theoretical point of view, in vivo labeling of red cells in small laboratory animals is the optimal, least damaging method of labeling without extracorporeal handling of red cells and without any influence on hematopoiesis by infusion of larger blood volumes.

Since minimal quantities (1–3 µl) of blood are needed for analysis of labeled red cells, an enhancing effect on hematopoiesis by blood loss – as suggested in

a previous paper [6] – can be excluded. Therefore, in vivo biotinylation is an excellent model for studying survival of red cells or hematopoiesis in small laboratory animals. Furthermore, survival slopes obtained after in vivo labeling are a good basis for comparison with survival slopes of red cells labeled by different procedures.

Short-term studies of transfused biotin-labeled red cells

Our short-term studies show that percentages of labeled red cells are within the predicted range and that an early decrease of labeling does not occur. In contrast, biotin-labeled red cells reach the highest values 2 or 3 h after injection. The increase of label shortly after transfusion can probably be explained by hemoconcentration after infusion of a 300-μl volume (=25% of the total blood volume). So we found 2-h values more adequate for the 100% reference.

Life span of biotin-labeled mouse red cells

The values and slopes vary between individual mice, which is in good agreement with the results of Slezak and Horan [15], who used the fluorescent lipophilic probe PKH-3 in rabbits. However, the general course of the slopes is similar. Calculated mean cell life spans L of mouse red cells are close to the 40.7±1.9 days reported by van Putten [13] using $DF^{32}P$ or the mean half-life of 8 days for old or of 12 days for young Balb/c mice reported by Magnani et al. [12], who used ^{51}Cr labeling of red cells and assumed a logarithmic slope of cell survival. The slopes are neither simple linear functions of time, as supposed for a strict age-related elimination of red cells, nor are they pure linear functions, as assumed for a random destruction of red cells. Table 2 shows that for mice with in vivo labeled transfused red cells the linear function obviously fits better with the obtained values.

For mice with in vitro labeled transfused red cells as well as for mice with in vivo labeled red cells we can see no uniform difference between the two models (Table 1 and 3). Our data show that a linear function is overlapped by a logarithmic function, where the components of both are unknown. During the first weeks the decrease of labeled red cells seems to be linear, but at the end of observation a logarithmic curve fits better with the data. Only 3 μl of blood were sufficient for FACS analysis. But different bleeding after the taking of blood samples may influence the survival slope of individual mice. This could be the reason for the "more logarithmic" slopes of some mice, and it is in agreement with the normal survival slopes as reviewed by Bentley [3].

We followed up the biotinylated red cells until they disappeared completely. Figures 3, 5, and 6 show that most of the mice had quite low values for a longer period, from about days 30–40 on, which produced a long curved "tail". Therefore, we calculated additionally the T_{50} survival times without the values of the "tail". When we neglected all the labeled red cell percentages below 5%, the calculated T_{50} values in the linear as well as in the logarithmic model came closer to the actually obtained values than when all the values of the slope tail were included (Table 4).

Table 4. T_{50} survival times of the three different experiments using all the obtained values or neglecting values below 5%

	Linear model 1		Log model 2		Actual obtained T_{50} values	
	T_{50}[a]	T_{50}[b]	T_{50}[a]	T_{50}[b]	Min	Max
In vivo labeled $n=4$	23.6±1.4	17.3±3.3	8.6±2.6	10.6±2.7	11	19
In vivo labeled and transfused $n=3$	24.1±1.4	22.7±2.1	10.9±2.2	16.1±3.2	20	25
In vitro labeled and transfused $n=6$	24.0±1.8	18.8±4.1	9.1±1.7	11.7±3.4	8	22
Mean	23.9±1.5	19.2±3.9	9.4±3.6	12.4±3.6		

[a] Mean and standard deviation when all the obtained values are included

[b] Mean and standard deviation when all values obtained below 5% are neglected

The conclusion from our experiment is that biotin is an ideal nonradioactive substance for labeling red cells in vitro and in vivo.

Acknowledgements. We thank Mrs. W. Norton for her technical assistance, Dr. N. Shearman for his critical review, and Mrs. S. Donhauser and Mrs. B. Engelbrecht for typing the manuscript.

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