Ann Hematol (1991) 63: 214–217

Annals of
Hematology

Original article

Direct in vivo biotinylation of erythrocytes as an assay for red cell survival studies*

G. Hoffmann-Fezer[1], H. Maschke[1], H. J. Zeitler[2], P. Gais[3], W. Heger[1], J. Ellwart[4], and S. Thierfelder[2]

[1] Institut für Immunologie, GSF
[2] Institut für klinische Hämatologie, GSF
[3] Institut für Strahlenschutz, GSF
[4] Institut für experimentelle Hämatologie, GSF

Received March 1, 1991 / Accepted July 16, 1991

Summary. Direct in vivo labeling of erythrocytes with biotin is shown as a method for estimation of red cell survival as well as of enrichment of young or aged erythrocytes. Two succinimide esters (biotin-N-hydroxysuccinimide ester [BNHS], caproylamidobiotin-N-hydroxysuccinimide ester [C-BNHS] were used for biotin labeling of erythrocytes. With improved syntheses, pure BNHS (mp, 212°–214° C) and the spacered intermediate for C-BNHS, 6-(biotinylamide) hexanoate (mp, 225°–226° C) were obtained in an overall yield of 86%; the yield of C-BNHS (mp, 167°–169° C) was 68%. When three doses of 1 mg C-BNHS are injected intravenously into mice at 24-h intervals, all the red cells are biotin labeled. The rate of red cell production as well as the life span of red cells can be measured without any effect on erythropoiesis or damage by red cells in vitro. The survival curve seems to be linear, with 2.5%–3.3% disappearance of biotin-labeled red cells daily. In mice, in vivo biotin labeling avoids damaging red cells by in vitro procedures and does not influence the steady state of erythropoiesis by hypertransfusion. Therefore, in vivo biotin labeling is a very useful method for determining red cell survival time in small animals.

Key words: Biotin labeling – Red cell survival

Introduction

Survival of red cells is usually determined with random labeling methods using chromium 51, ferrum 59, technetium 99, indium 111, or diisopropylphosphorofluoridate tagget with phosphorus 32 [3, 15]. ^{51}Cr is the most popular radiometric method [8]. None of these generally accepted techniques can completely fulfill the requirements for an easy and exact determination of the mean red cell life span [17]. Nonradioactive methods available are the Ashby method [1] and a recently developed fluorescent labeling with the fluorescent lypophilic probe PKH 3 [17]. We want to present in vivo biotin labeling as an alternative, nonradioactive approach for irreversible labeling of red cells. Biotin labeling of red cells in vitro has been used [19] in rabbits for survival studies and in man for measuring the total volume of red cells [4]; more recently, in vivo biotin labeling in rabbits was used for enrichment of aged erythrocytes [6]. We found that in vivo labeling is appropriate for determination of red cell survival or red cell production.

Address for correspondence: G. Hoffmann-Fezer, Institut für Immunologie, GSF, Marchioninistrasse 25, W-8000 München 70, FRG

* Supported by DFG (Ho 779/4–1)

Material 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.

Synthesis of biotin-N-hydroxysuccinimide ester (BNHS)

An improved synthesis of BNHS was achieved by applying the following procedures: before use, dimethylformamide (analytical grade/for residue analysis, Merck, Darmstadt, FRG) and 2-propanol (analytical grade, Merck, Darmstadt, FRG) were dried for some days with a 4-Å molecular sieve (Merck, Darmstadt, FRG). D(+)-Biotin (Serva, Heidelberg, FRG), N-hydroxysuccinimide, and N,N′-carbonyldiimidazole (both from Merck, Darmstadt, FRG) were stored in vacuo over KOH. The melting points (mp) were obtained using a Leitz-Mikroskop-Heiztisch and are uncorrected.

Twenty grams (81.9 mmol) of D(+)-biotin were dissolved in 450 ml dry dimethylformamide at 80° C in a round-bottom flask. To the magnetically stirred solution 14.6 g (90 mmol) of N,N′-carbonyldiimidazole in 350 ml dry dimethylformamide were added. The temperature was kept at 80° C until the evolution of carbon dioxide had ceased. Then, the clear solution was stirred for 3 h at room temperature. The precipitated biotinylimidazolide was converted into the N-hydroxysuccinimide ester (BNHS) by adding 9.9 g (86 mmol) of N-hydroxysuccinimide in 350 ml dry dimethylformamide and stirring the suspension (N_2 atmosphere; flask sealed with Parafilm) for 12 h, during which time the precipitation dissolved completely. After the dimethylformamide had been removed

at reduced pressure (rotary evaporator) the resulting oil was treated with dry 2-propanol and the solvent was distilled off in vacuo. The residue was boiled for some minutes with 300 ml of dried 2-propanol (magnetically stirred) and the suspension was cooled to 0° C. The precipitated BNHS was dried in vacuo (yield, 27.6 g = 98.8%; mp = 206°–209° C). A second extraction with 4000 ml of boiling 2-propanol yielded, after cooling, 24.0 g (85.9%; mp = 209°–212° C) of BNHS, which was then recrystallized from dry 2-propanol (275 ml 2-propanol per g BNHS) to give 21.8 g (78.2%) of pure BNHS (mp = 212°–214° C).

Synthesis of caproylamidobiotin-N-hydroxysuccinimide ester (C-BNHS)

To a magnetically stirred solution of 3.4 g (10 mmol) of biotin-N-hydroxysuccinimide ester (BNHS) in 50 ml of dry dimethylformamide in a round-bottom flask, 40 ml of 0.1 *M* aqueous sodium hydrogencarbonate containing 1.3 g (10 mmol) 6-aminocaproic acid (analytical grade; Merck, Darmstadt, FRG) were added. After 5 h the solvents were removed at reduced pressure (rotary evaporator) and the oily residue was treated with 200 ml of aqueous citric acid (100 g/l) at 40° C. The 6-(biotinylamido)hexanoate was separated by filtration and washed with de-ionized water. After drying in a vacuum desiccator over KOH at 80° C overnight, the yield was 3.27 g (91.5%) and the melting point was 220°–222° C. The pure intermediate was obtained by recrystallization from 280 ml of aqueous 2-propanol (isopropanol-water = 8 + 2) in an overall yield of 86.0% (3.08 g) and with a melting point of 225°–226° C.

Three grams (8.4 mmol) of 6-(biotinylamido)hexanoate were dissolved in 200 ml of dried dimethylformamide at 80° C in a round-bottom flask. After addition of 1.6 g (9.5 mmol) of N,N'-carbonyldiimidazole in 35 ml of dry dimethylformamide, the temperature was held until the evolution of CO_2 had ceased.

The clear solution was then stirred for 3 h at room temperature and the intermediate imidazolide was converted into the N-hydroxysuccinimide ester (C-BNHS) by addition of 1.0 g (8.7 mmol) N-hydroxysuccinimide in 35 ml of dried dimethylformamide and stirring (nitrogen atmosphere; Parafilm-sealed flask) at room temperature for 12 h. Then the solvent was distilled off at reduced pressure (rotary evaporator) and the oily residue was treated with dry 2-propanol (50 ml) at approximately 70° C. After the solvent had been removed, the precipitation was recrystallized from 80 ml of boiling 2-propanol. The caproylamidobiotin-N-hydroxysuccinimide ester (C-BNHS) was obtained in a 68% yield (2.6 g) after drying at 90° C in vacuo for 12 h and had a melting point of 167°–169° C.

The succinimide esters (Parafilm-sealed tubes) were stored in a desiccator, or over P_2O_5 or KOH at +4° C (refrigerator), and adapted to room temperature 3 h before use. After 6–12 months, the succinimide esters may reveal a lower activity in biotin labeling of cells. To fully restore their coupling activity, the esters were recrystallized from boiling 2-propanol (dried over 4-Å molecular sieve).

Biotin labeling of erythrocytes

Three daily 1-mg doses of C-BNHS dissolved in 50 µl dimethylformamide and 250 µl PBS were injected intravenously (i.v.) on consecutive days.

Recovery of biotin-labeled red cells

About 20–30 µl of blood were withdrawn from mouse tail veins using a hematocrit capillary 60 min and 1, 4, 7, 9, 11, 13, 15 days, or 60 min and daily between days 1–6 after the last i.v. injection. The blood was diluted in MEM and 2×10^4 erythrocytes each were bound to poly-L-lysine spots on multispot slides (plls) prepared according to Bross [2], modified by Kranz et al. [9, 10, 11]. Samples of biotinylated or unlabeled red cells were used as positive and negative controls. The red cells were incubated with alkaline phosphatase-labeled avidin (Avi-aP, Sigma). Alkaline phosphatase activity was revealed according to Burstone [3], modified using fast blue BB (Sigma) and naphthole AS phosphate (Sigma). Detection of the percentage of labeled erythrocytes was performed by microscopic evaluation, as well as by semiautomatic image analysis.

Semiautomatic image analysis

With the semiautomatic image analysis method (7), ten microscopic images were randomly selected from each of the fields of a plls. The image sizes are 256×256 µm (25× objective, n. ap. 0.53). Each image contains an average of up to 150 red cells. They were scanned by a TV microscope (IBAS Kontron, Zeiss, UEM) at 550 nm wavelength, digitized with a pixel separation of 0.5 µm, segmented and preprocessed for separation of labeled and non-labeled red cells by a thresholding algorithm in respect to optical density measurements. The threshold must be adapted interactively to each field of the plls and will not be changed between the measurements of the ten images.

Results

BNHS injected intravenously did not sufficiently label red cells, but a single i.v. injection of 1 mg C-BNHS weakly labels most red cells. Three daily doses of 1 mg are sufficient to label distinctly 100% of erythrocytes. After incubation with Avi-aP and enzymecytochemical staining for aP, labeled red cells are blue and can be distinguished clearly from unlabeled, unstained erythrocytes. The method of semiautomatic image analysis also discriminates between labeled and unlabeled cells.

Figure 1 shows decreasing mean percentages of biotinylated red cells over 6 or 15 days following the last biotin injection. Both slopes are almost linear but a little steeper from about day 3 on.

Values of labeled red cells of another experiment are depicted in Fig. 2. Figure 2a shows labeled red cells evaluated by microscopic enumeration and the regression line y = 100.7 − 3.3 x, whereas in Fig. 2b values of labeled erythrocytes were obtained by semiautomatic image analysis (regression line y = 100.5 − 2.5 x).

Discussion

Our previous experiments to determine spectrophotometrically the alkaline phosphatase activity bound to bio-

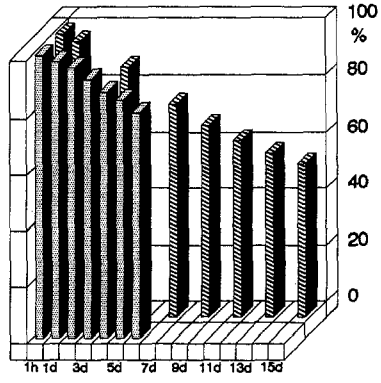

Fig. 1. Decreasing percentages of biotinylated red cells during days 1–15 (*back*) and on consecutive days 1–6 (*front*)

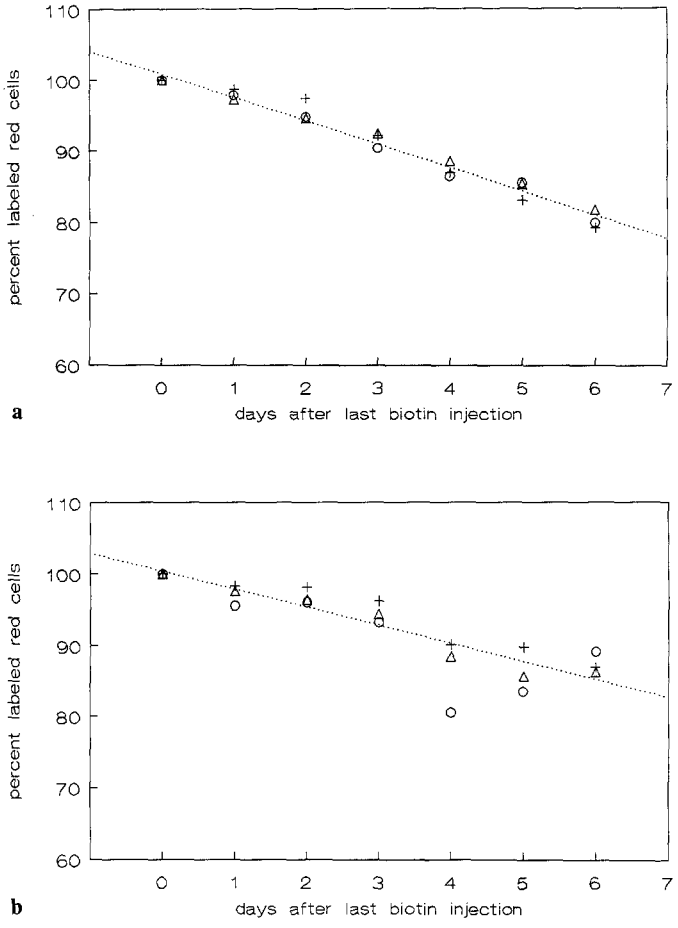

Fig. 2a, b. Comparison of values of labeled red cells and regression lines of the same assay evaluated (**a**) by microscopic enumeration; (**b**) by semiautomatic image analysis

tin-labeled erythrocytes were not successful. Unlabeled red cells bound very small amounts of alkaline-phosphatase-labeled avidin. Therefore, a summary enzyme reading was not able to discriminate between small percentages of distinctly labeled red cells and the bulk of unbiotinylated erythrocytes, which showed a very faint unspecific signal. Therefore, we decided to use a cytochemical method and microscopic numeration for measuring labeled cells. We also performed semiautomatic image analysis for a faster and more convenient measuring of labeled red cells.

Application of a synthetic spacered biotin ester (C-BNHS)

Direct in vivo labeling of primary amino groups of surface proteins of red cells (as recently described also by Dale et al. [6]) and the use of a spacered biotin ester (caproylamido-biotin-N-hydroxysuccinimide ester, C-BNHS) has the advantage of being an easier labeling procedure than the original method of Suzuki et al. [19], who labeled rabbit erythrocytes with succinylated BSA followed by N-hydroxysuccinimidobiotin. Definite biotinylations are dependent on the purity of the biotin esters used. Purchasable biotin esters often contain impurities (inconstant melting and/or coupling activities) and are very expensive for large-scale experiments.

With the earlier-described synthesis of BNHS [9], we obtained BNHS fractions with a melting range of 185°–206° C (210° C in [9]). We thus improved the chemical synthesis for the biotin esters used in our studies. Following the procedure described here, we obtained the BNHS in a good yield and with a constant melting point. The spacered ester, C-BNHS, was analogously synthesized from the purified and crystallized intermediate, 6-(biotinylamido)hexanoate. It showed a constant melting point that was approximately 20° C higher than reported by Costello et al. [5].

In our experiment biotin is attached via the spacer arm to the red cell. We never observed large numbers of echinocytes as described by Suzuki et al. [19] after incubation with NHS Biotin.

Interpretation of data

The results in Figs. 1 and 2 show an almost linear disappearance of labeled red cells, with a small variation of values in Fig. 2a. The blood sample drawn daily for determination of labeled red cells was about 20 μl each. This corresponds to 1.6% of the 1.260-ml estimated blood volume of a female CBA mouse [18]. After some days this loss of blood may induce a slight activation of erythropoiesis. This may explain the negligible steeper slope from day 3 on (Fig. 1). When a linear survival slope is suggested the daily loss of labeled red cells or increase of unlabeled cells calculated from the regression of Fig. 2a is 3.3%. This would mean a half survival time (T_{50}) of mouse red cells of 15.4 days. This means that 50% of red cells survive at day 15. Using the regression of Fig. 2b, the daily loss of labeled red cells is 2.5% and the T_{50} of red cells is 20.2 days. This second value, obtained by semiautomatic image analysis, is very close to the mean survival time of 40.5 ± 1.9 days reported by van Putten [16] using DFP^{32}, and to the T_{50} of 8 days for old, or of 12 days for young male Balb/c mice reported by Magnani et al. [14], who used ^{51}Cr labeling of red cells and assumed a logarithmic slope of red cell survival. Due to the similarity of our results with those obtained with radioactive labeling we chose to dispense with the radioactive methods.

Evaluation of biotin-labeled red cells

In our study the biotin-labeled cells were evaluated by alkaline-phosphatase-labeled avidin and subsequent cytochemistry. Interpretation used visual criteria for distinguishing between labeled and unlabeled red cells. This technique is obviously advantageous compared with avidin-labeled polystyrene beads used by others [6, 19], and obviously also compared with the FITC-labeled streptavidin method of Cavill et al. [4]. These authors were not able with FACS analysis to find labeled red cells

7 days after transfusion of in vitro biotin-labeled erythrocytes, although they were able to use the biotin labeling of red cells for determining the total volume of red cells.

Use of semiautomatic image analysis

We wondered if semiautomatic image analysis would work more rapidly and precisely than microscopic enumeration in evaluating labeled red cells. As demonstrated in Fig. 2, a higher variation of values occurs in semiautomatic image analysis, although this procedure requires less time. Further studies using in vivo biotin-labeled red cells for transfusion and follow-up of labeled red cells are in progress.

Conclusion. The major advantages of our technique of direct in vivo labeling with biotin are (a) that red cells must not be handled and potentially impaired in vitro and (b) that there can be no inhibitory influence on hemopoietic activity due to a hypertransfusion effect of injected labeled red cells [12, 13, 20]. Furthermore, there is no reutilization of biotin. The sole disadvantage is that red cells can be labeled in vivo only in small laboratory animals, owing to the high cost of biotin ester.

Acknowledgements. We thank Mrs. S. Donhauser for typing the manuscript.

References

1. Ashby W (1919) The determination of the length of the life of transfused blood corpuscles in man. J Exp Med 29: 267–281
2. Bross KJ (1980) Nachweis von Zellmembranantigenen mit einer Immunoperoxidase-Objektträgermethode. Habilitationsschrift, Universität Freiburg/Brsg
3. Burstone MS (1959) New histochemical techniques for the demonstration of tissue oxidase (cytochrome oxidase). J Histochem Cytochem 7: 112–122
4. Cavill I, Trevett D, Fisher J, Hoy T (1988) The measurement of the total volume of red cells in man: a non-radioactive approach using biotin. Br J Haematol 70: 491–493
5. Costello SM, Felix RT, Giese RW (1979) Enhancement of immune cellular agglutination by use of the avidin-biotin system. Clin Chem 25: 1572–1580
6. Dale GL, Norenberg SL (1990) Density fractionation of erythrocytes by Percoll/hypaque results in only a slight enrichment for aged cells. Biochim Biophys Acta 1036: 183–187
7. Gais P, Rodenacker K (1987) Hard- and software requirements of a multipurpose image analysis system. In: Burger G, Ploem JS, Goerttler K (eds) Clinical cytometry and histometry. Academic, London, pp 36–38
8. International Committee for Standardization in Hematology (1982) Recommended method for radioisotype red-cell survival studies. Br J Haematol 45: 659–666
9. Jasiewicz MJ, Schoenberg DR, Mueller GC (1976) Selective retrieval of biotin-labeled cells using immobilized avidin. Exp Cell Res 100: 213–217
10. Kranz B, Thierfelder S (1986) Improved detection of terminal transferase (TdT): the use of detergents on glutaraldehyde fixed non-dehydrated cells prevents denaturation and diffusion artifacts. Leukemia Res 10: 1041–1049
11. Kranz B, Thiel E, Thierfelder S (1989) Immunocytochemical identification of meningeal leukemia and lymphoma: Poly-L-lysine-coated slides permit multimarker analysis even with minute cerebrospinal fluid cell specimens. Blood 73: 1942–1950
12. Krzymowski T, Krzymowska H (1982) Studies on the erythropoiesis-inhibiting factor in the plasma of animals with transfusion polycythemia. Blood 19: 38–44
13. Lindemann R (1971) Erythropoiesis-inhibiting factor (EIF). I. Fractionation and demonstration of urinary EIF. Br J Haematol 21: 623–631
14. Magnani M, Rosse L, Stocchi V, Cucchiarini L, Piacentini G, Fornaini G (1988) Effect of age on some properties of mice erythrocytes. Mech Ageing Dev 42: 37–47
15. Mollison PL (1983) Blood transfusion in clinical medicine. Blackwell Scientific, Oxford
16. Putten LM Van (1958) The life span of red cells in the rat and the mouse as determined by labeling with DFP^{32} in vivo. Blood 13: 789–794
17. Slezak SE, Horan PK (1989) Fluorescent in vivo tracking of hematopoietic cells. I. Technical considerations. Blood 74: 2172–2177
18. Sluiter W, Hulsing-Hesselink E, Elzenga-Claasen I, Van Furth R (1985) Method to select mice in the steady state for biological studies. J Immunol Methods 760: 135–143
19. Suzuki T, Dale GL (1987) Biotinylated erythrocytes: in vivo survival and in vitro recovery. Blood 70: 791–795
20. Whitcomb WH, Moore MZ (1965) The inhibitory effect of plasma from hypertransfused animals on erythrocyte iron incorporation in mice. J Lab Clin Med 66: 641–651