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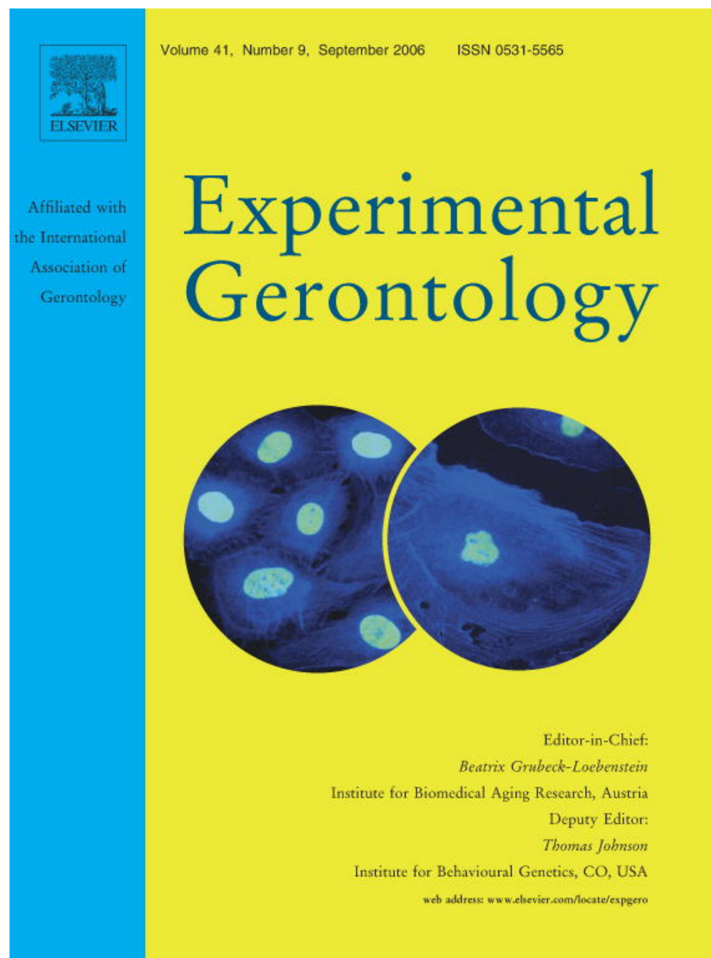

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Assessment of survival of aging erythrocyte in circulation and attendant changes in size and CD147 expression by a novel two step biotinylation method

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Abstract

Three intravenous injections (1 mg each) of biotin-X-NHS (BXN) given at 24 h intervals labeled all circulating erythrocytes with biotin in C57Bl/6 mice. After 5 days, administration of another i.v. injection of BXN (0.6 mg) resulted in the labeling of erythrocytes released in blood circulation after the first biotinylation step, with a lower intensity of biotin. The older erythrocyte population with high intensity of biotin (biotin^{high} population) and the later population of newly formed erythrocytes with lower intensity of biotin (biotin^{low} population) could be stained with streptavidin-APC (SAv) and identified by flow cytometry. Using the double biotinylation technique, we could examine the survival and age related changes in biotin^{low} population of erythrocytes that was released in circulation during a defined time period (5 days). Our results indicate that the percentage of Biotin^{low} erythrocytes in circulation remained static for 10 days after the second biotinylation step and than started to decline steadily with time. Mean fluorescence intensity of biotin label on surviving biotin^{low} population of erythrocytes however remained stable. These results suggest that after 15 days of release in blood, erythrocytes may undergo random destruction. Furthermore, forward scatter as well as CD147 expression of Biotin^{low} population also declined with age. Double biotinylation technique described in this communication offers an easy method for tracking age related changes in populations of erythrocytes released in circulation during a defined period of time.
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Keywords: Erythrocyte turnover; Biotin; Murine; Forward scatter; CD147; Reticulocytes; Thiazole orange; Erythrocyte aging; RBC; Blood circulation

1. Introduction

Life span of circulating human and murine erythrocytes has been estimated to be 120 and 50 days, respectively (Goodman and Smith, 1961; Horky et al., 1978; Piomelli and Seaman, 1993; Deiss, 1999), indicating that roughly 1% and 2% of circulating erythrocytes are destroyed each day in humans and mice, respectively. Bulk of the erythrocyte destroying activity takes place in the reticulo-endothelial system (RES) in spleen, bone marrow and liver (Clark, 1988). What feature(s) of circulating erythrocyte earmarks them for destruction is however not clearly understood. In general it is believed that as erythrocytes age in circulation, many changes occur in these cells that are recognized by the RES resulting in phagocytosis of the senescent erythrocytes (Kiefer and Snyder, 2000; Stadtman et al., 2005). Band 3 protein modifications may take place in erythrocyte membrane as a result of accumulated oxidative insults and altered band 3 protein may fix naturally occurring antibody and complements (Kay, 2004; Arese et al., 2005). Changes similar to apoptosis like the extrusion of phosphatidylserine in erythrocyte membrane may also occur in aged erythrocytes making them susceptible to phagocytosis (Bosman et al., 2005).

While many types of changes are associated with the process of aging of erythrocytes, such studies are hampered by

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lack of a method of recognizing and isolating erythrocytes of defined age group in circulation. Separation of aged erythrocytes on the basis of buoyant density has been suggested, but the efficacy of the method has not been established (Dale and Norenberg, 1990). Suzuki and Dale (1987, 1988) proposed a method for biotinylation of erythrocytes and demonstrated that when infused in vivo, biotin label on erythrocytes was stable in circulation. Hoffmann-Fezer et al. (1991, 1993) further improved the method by demonstrating that circulating erythrocytes could be biotinylated in vivo and the label could be used to assess the survival of erythrocytes and isolation of erythrocyte populations enriched in aged cells. The in vivo biotinylation method has been used to assess changes during aging of circulating erythrocytes (Rettig et al., 1999) and to demonstrate decreased survival of erythrocytes in murine sickle cell anemia (De Jong et al., 2001) and malarial anemia (Evans et al., 2006). The one step biotinylation method however has limitations. At the time of in vivo labeling of erythrocytes with biotin, all erythrocytes are labeled irrespective of their age in circulation. Later as fresh erythrocytes enter the circulation, an older biotinylated population and another non-biotinylated younger population can be clearly identified in blood. The method cannot however be used to selectively label erythrocytes of a defined age group.

In the present communication, we propose a novel technique involving two in vivo biotinylation steps that enables us to tag with biotin a population of erythrocytes entering blood circulation over a defined time period, and later to follow this population as it ages in circulation. In this technique, the first conventional biotinylation step ensures a complete high intensity labeling of all circulating erythrocytes (biotin^{high} population) (Hoffmann-Fezer et al., 1991, 1993). This is followed after 5 days with a second in vivo biotinylation step with a single lower dose of the biotinylation agent. The second step labels the newly formed erythrocytes with a lower intensity of biotin (biotin^{low} population). Both high and low biotinylated erythrocyte populations can be stained with streptavidin-APC and clearly identified by flow cytometry. Changes in the biotin^{low} population can be studied as this population of erythrocytes ages in circulation. By using this technique, we have studied the survival kinetics of erythrocytes as well as the kinetics of changes in forward scatter and CD147 expression on age defined population of erythrocytes. Our results suggest that after 10–15 days of entering the blood stream, erythrocytes may become susceptible to random killing. An age related decline in size and CD147 expression has also been demonstrated.

2. Materials and methods

2.1. Mice

Inbred C57BL/6 female mice (8–12 weeks old, 20–25 g body weight) were used throughout this study. Animals were bred and maintained in the animal house facility at JNU, New Delhi or obtained from the National Institute of Nutrition, Hyderabad. The animals were housed in positive-pressure air conditioned units (25 °C, 50% relative humidity) and kept on a 12 h light/dark cycle. Water and mouse chow were provided *ad libidum.* All the experimental protocols were approved by JNU Institutional Animal Ethics Committee and performed accordingly.

2.2. Reagents

Sources of reagents were: biotin-X-NHS ester (Calbiochem La jolla, CA), streptavidin fluoresecin-isothiocynate (SAv FITC), streptavidin allophycocyanin (SAv APC) were from BD Biosciences (San Diego, CA). Anti-mouse CD147-FITC was from the E-Biosciences (San Diego, CA). Fetal bovine serum was obtained from Hyclone (South Logan, Utah). Thiazole orange, dimethylformamide (DMF) and other reagents were from Sigma-Aldrich (India).

2.3. Biotin labeling

Mice were given three daily i.v. injections of 1 mg of biotin-X-NHS Ester (BXN) dissolved in 20 μl of DMF and 250 μl of PBS. For the second biotinylation step, mice were given 0.6 mg of BXN dissolved in 12 μl of DMF and 250 μl of PBS, 5 days after the last injection of the first step biotinylation.

2.4. Flow cytometry

Blood was collected in EDTA (5 mM/ml) and washed three times with ice cold normal saline containing 10 mM Hepes buffer (pH 7.4) and 1% FBS. For flow cytometric studies, one million cells were stained with streptavidin-FITC or APC, and/or other antibodies as recommended by the manufacturers. For thiazole orange staining, cells were incubated with dye (50 ng/ml) for 30 min at room temperature and washed. Stained erythrocytes were immediately analyzed on FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cell Quest software for acquisition and analysis. A minimum of 10,000 events were recorded for each sample.

2.5. Statistical analysis

Statistical analysis was done using Sigma plot software. Data are presented as means ± SD. Significant values were calculated using Students-*t*-test.

3. Results

3.1. In vivo delineation of erythrocytes of three different age groups by a double biotinylation procedure

Three daily i.v. injections (1 mg/day) of biotin-X-NHS (BXN) resulted in biotinylation of all circulating

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erythrocytes in mice. Biotinylated erythrocytes stained with streptavidin-APC could be revealed by flow cytometry. Results in Fig. 1A show that 99.4% of erythrocytes (Box-X in Fig. 1A) were biotinylated after this biotinylation step. Five days after the last injection of BXN about 10% of erythrocytes were found to be free of biotin label and represented erythrocytes freshly released into blood after BXN injections (Box-Z in Fig. 1B). At this time a single lower dose (0.6 mg) of BXN resulted in labeling of the fresh unlabeled erythrocyte population albeit with a lower stain intensity (Box-Y in Fig. 1C). Results in Fig. 1D–F show that after 5, 25 and 50 days of second biotinylation step, three discreet erythrocyte populations could be identified in the blood based upon the intensity of labeling with biotin. The most aged erythrocytes population biotinylated during the first step of biotinylation, had the highest intensity of stain (Box-X, designated biotin^{high} population). Erythrocytes biotinylated by the second dose of BXN had an intermediate intensity of biotin (Box-Y, to be designated biotin^{low} population) and represented erythrocytes from the population released in blood between first and second biotinylation step. Unlabeled erythrocytes in Box-Z represented fresh cells released into blood circulation after the second administration of BXN. Thus by using this technique, it is possible to gate on erythrocytes of three distinct age groups. Furthermore, the mean intensities of biotin label remained steady on biotin^{high} and biotin^{low} populations of erythrocytes until 50 days after the second biotinylation step (Fig. 2).

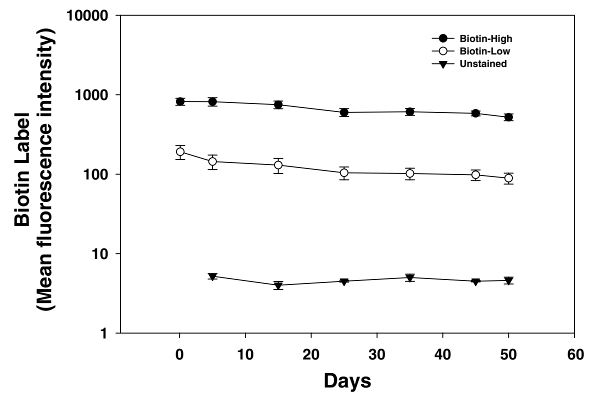

Fig. 2. Stability of biotin label on biotin^{high} and biotin^{low} erythrocytes aging in blood circulation. Mouse erythrocytes were labeled with biotin by the two step procedure as described in legend to Fig. 1. Intensities of biotin label (mean fluorescence intensity of SAv-APC stain) on biotin^{high}, biotin^{low} and biotin^{-} populations of erythrocytes were determined 0, 5, 15, 25, 35, 45 and 50 days after second biotin dose. Each point on the graph represents mean ± SD of observations on five mice.

3.2. Association of reticulocytes with the freshly released population of erythrocytes

In order to confirm that the biotin negative erythrocytes in Fig. 1B and biotin^{low} population in Fig. 1C represented erythrocytes freshly released in the blood after the first BXN administration, erythrocyte preparations were counterstained with thiazole orange, a dye that stains nucleic acids. Results in Fig. 3A show that after 5 days of first biotinylation step, reticulocytes (thiazole orange positive

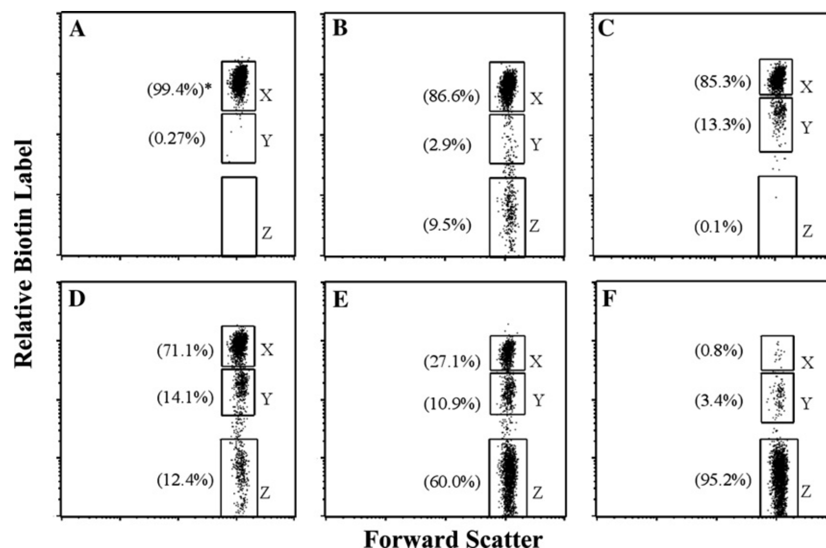

Fig. 1. Demarcation of three discreet erythrocyte populations in blood using a two step biotinylation procedure. C57Bl/6 mice were administered intravenously three daily doses of 1 mg biotin-X-NHS Ester (BXN) [first biotinylation step]. After a 5 day rest, a single additional dose of 0.6 mg BXN was administered [second biotinylation step]. Blood was collected at different time points and distribution of biotin label on erythrocytes was examined by staining the cells with streptavidin-APC and flow cytometric analysis as described in methods. Biotin label on circulating erythrocytes was examined 2 h (panel A) and 5 days (panel B) after the first step of biotinylation, and 2 h (panel C), 5 days (panel D), 25 days (panel E) and 50 days (panel F) after the second step of biotinylation. Erythrocyte populations in boxes X, Y and Z represent biotin^{high}, biotin^{low} and biotin^{-} populations of erythrocytes. Values in parentheses represent percentage of cells in different boxes.

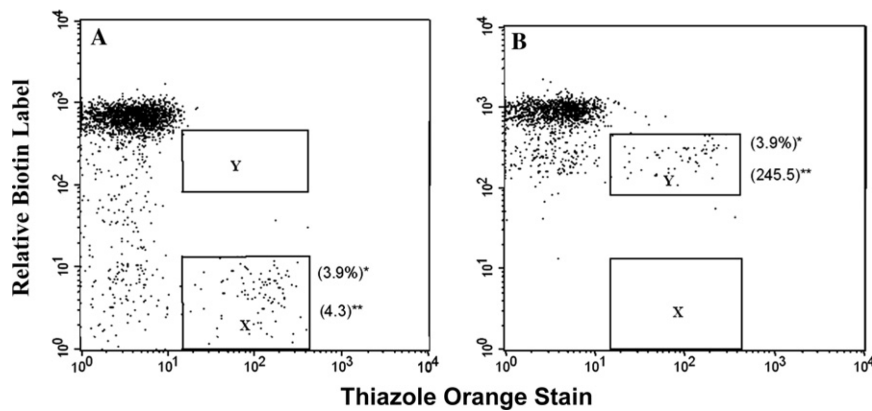

Fig. 3. Association of thiazole orange stained reticulocytes with the newly formed erythrocytes. Circulating erythrocytes were labeled with biotin by the two step biotinylation procedure as described in legend to Fig. 1. Erythrocytes obtained immediately before (left panel) and after (right panel) the second step of biotinylation, were stained with SAv-APC as well as thiazole orange (TO) and analyzed on flow cytometer. Cell populations in boxes X and Y represent TO stained reticulocytes associated with biotin negative and biotin^{low} populations of erythrocytes, respectively. Values in parentheses show the percentage of TO^{+} reticulocytes* and biotin label (mean fluorescence intensity of SAv-APC stain)** on boxed populations of reticulocytes.

population) were associated with biotin negative population of erythrocytes and comprised about 4% of the whole erythrocyte population. After second step of biotinylation, the reticulocyte population migrated along with the biotin^{low} population (Fig. 3B).

3.3. Survival kinetics of mouse erythrocytes in vivo

The procedure described above allowed us to gate on an erythrocyte population with defined age group i.e. biotin^{low} population, and study its properties as this population of erythrocytes ages in circulation. Results in Fig. 4 show the survival of biotin^{low} population of erythrocytes as a function of the age and indicate that the proportion of biotin^{low} erythrocytes remained steady till 10 days after the second biotinylation step and fell constantly thereafter over a period of 50 days, even though the rate of decline was relatively lower during first phase of 10–35 days and increased thereafter.

Erythrocyte size is known to decrease with age (Vomel et al., 1980; Thompson et al., 1984; Waugh et al., 1992). We measured changes in forward scatter, a flow cytometry parameter related to cell size, of biotin^{low} population of erythrocytes as a function of age. Results in Fig. 5 show that the forward scatter fell significantly till biotin^{low} population reached 20 days of age. A second phase of fall in

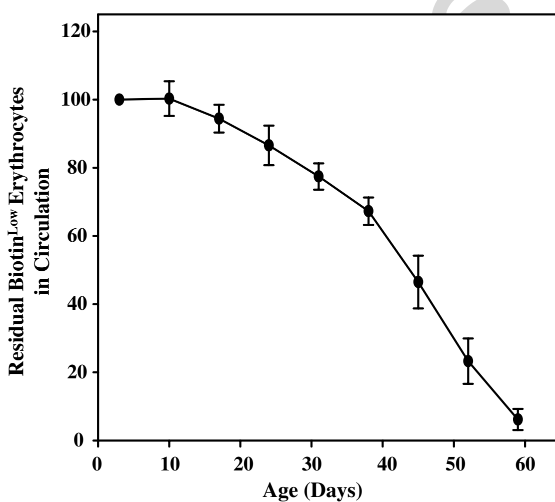

Fig. 4. Survival kinetics of erythrocytes in the blood circulation. Mouse erythrocytes were biotin labeled by two step biotin method as described in legend to Fig. 1. Blood samples were collected 0, 7, 14, 21, 35, 42, 49 and 56 days after second biotin dose and concentration of biotin^{low} erythrocytes as percentage of all erythrocytes was determined. Each point on the graph represent mean ± SD of observations on seven mice.

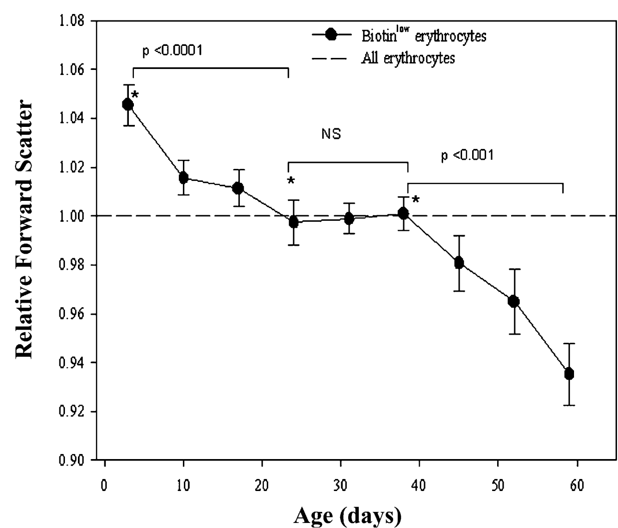

Fig. 5. Age dependent changes in the forward scatter of erythrocytes. Mouse erythrocytes were labeled with biotin by the two step procedure as described in legend to Fig. 1, and forward scatter of biotin^{low} population was examined 0, 7, 14, 21, 35, 42, 49 and 56 days after second biotin dose. Dotted line represents the mean forward scatter of all erythrocytes. Each point on the graph represent mean ± SD of observations on seven mice.

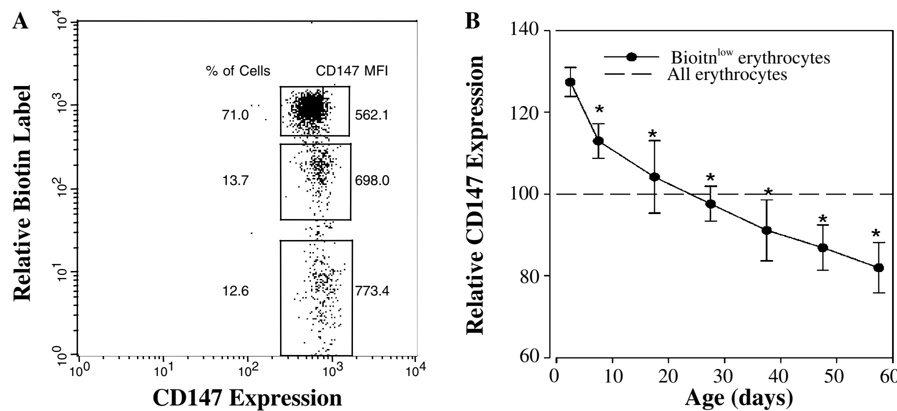

Fig. 6. Age dependent changes in CD147 expression as circulating mouse erythrocytes age. Mouse erythrocytes were labeled with biotin by the two step procedure as described in legend to Fig. 1. Five days after the second biotinylation step, erythrocytes were stained with SAv-APC and anti-CD147-FITC mab. Percentage of biotin^{high}, biotin^{low} and biotin^{-} populations of erythrocytes and the mean expression of CD147 on these populations was determined (panel A). CD147 expression on biotin^{low} population was examined 5, 15, 25, 35, 45 and 50 days after second biotin dose (panel B). Each point on the graph represent mean ± SD of observations on five mice. $* \ p < 0.005$ and 0.0002 for comparison of different time points with the first time point.

forward scatter occurred between ages 35 and 60 days. Overall the fall was about 10% over 60 days, but was highly significant statistically ($p < 0.0001$).

3.4. Change in CD147 expression on aging erythrocytes

CD147 is an adhesion protein expressed on erythrocytes that regulates the recirculation of erythrocytes through spleen (Coste et al., 2001). Changes in the expression of CD147 antigen as a function of age of erythrocytes were examined. Results in Fig. 6A indicate that the mean expression of CD147 on biotin negative population was the highest (mean fluorescence intensity = 773). It was significantly lower for biotin^{low} and biotin^{high} populations (MFI = 698 and 562, respectively). Fig. 6B shows that the CD147 expression on biotin^{low} population of erythrocytes declined progressively from the time when fresh erythrocytes entered the circulation. Overall a 35% decrease in CD147 expression was observed in the oldest erythrocytes as compared to the youngest.

4. Discussion

Circulating erythrocytes have a life span of 50–60 days in mice (Goodman and Smith, 1961; Horky et al., 1978; Piomelli and Seaman, 1993). Phagocytosis by macrophages in the reticulo-endothelial system in liver, bone marrow and spleen is considered to be the prime mechanism of destruction of erythrocytes in circulation. Several suggestions have been made about the changes in aging erythrocytes that may render them susceptible to phagocytosis (Bratosin et al., 1998; Kuypers and De Jong, 2004; Lutz, 2004; Bosman et al., 2005), though the factors that earmark erythrocytes for destruction are not as yet clearly understood. Such studies are hampered by lack of a good method to track an erythrocyte population of defined age group in experimental animals.

In vivo biotinylation of erythrocytes provided a good technique to study aging of erythrocytes in vivo (Suzuki and Dale, 1988; Hoffmann-Fezer et al., 1993). Three daily intravenous injections of biotinylation reagent were shown to label all erythrocytes in circulation (Hoffmann-Fezer et al., 1993). Fresh erythrocytes that appeared in blood after the biotinylation step were not biotinylated and therefore, biotin^{+} and biotin^{-} populations isolated from blood 5 days after the first biotinylation step, represented erythrocytes released into blood stream before and after the first biotinylation step. This technique could be used to study very old or very young erythrocytes (Suzuki and Dale, 1988; Christian et al., 1993) but could not track an age defined erythrocyte population in circulation.

Technique of double biotinylation described in the present study provides an easy method to label an erythrocyte population that enters blood circulation within a short defined time span, and track it down as it ages in vivo. This was achieved by following the first biotinylation step that labeled all erythrocytes with biotin, with a second low intensity biotinylation step after 5 days. Fresh erythrocytes released in circulation within this period of 5 days were labeled with a lower intensity of biotin. This band of biotin^{low} erythrocytes could then be tracked over a period of time for assessing age related changes. Second biotinylation step resulted in a small additional biotinylation of the biotin^{high} population because the latter population was already labeled to saturation by the first biotinylation step. This increase did not significantly change the position of biotin^{high} population on the flow cytometer scattergram since the biotin intensity axis is on a log scale. In this study the second biotinylation step was performed 5 days after the primary biotinylation, but this duration could be altered to tag erythrocytes freshly released in blood over any desired time period. The intensity of biotin label on erythrocytes was stable and the difference in biotin label on biotin^{high} and biotin^{low} populations of erythrocytes were

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sustained till the labeled erythrocytes were all together lost due to normal turnover (Fig. 2). Stability of biotin label on erythrocytes labeled by three i.v. injections of the biotinylation agent has been demonstrated before (Hoffmann-Fezer et al., 1991, 1993). Our results confirm this observation and further show that the biotin label was also stable on the biotin^{low} population of erythrocytes labeled with a subsequent administration of BXN at a lower dose.

Survival kinetics of circulating erythrocytes is of interest as it can be a pointer to the mechanism of erythrocyte destruction. Two extreme models have been considered about the destruction of erythrocytes. First, it is possible that erythrocytes are only destroyed when they reach a certain age in circulation and acquire certain crucial age dependent markers (Eadie and Brown, 1953; Clark, 1988). Alternatively, erythrocyte destruction could be a random process in which a certain fraction or erythrocytes are destroyed each day, irrespective of their age (Burwell et al., 1953; Eadie and Brown, 1953; Clark, 1988). If the first model is correct, the proportion of biotin^{low} population of erythrocytes would start to decline in the blood only when this population has aged sufficiently and remain constant before such time. On the other hand, if erythrocyte destruction is random, the proportion of biotin^{low} population of erythrocytes would fall from the very beginning as a function of the age. Our results indicate that the proportion of biotin^{low} erythrocytes remained constant for ten days and fell consistently over next 50 days (Fig. 4). Shape of the survival curve for biotin^{low} population of erythrocytes suggests that after an initial phase of about 10 days, freshly released erythrocytes may become susceptible to random killing. Thus the true picture of erythrocyte survival appears to be a combination of the two extreme models stated above. It is however interesting to note that the slope of the survival curve appears to be relatively low between 10 and 35 days and increased thereafter. Thus it is possible that selective destruction of older erythrocytes may also be a contributory factor in erythrocyte destruction.

We also utilized the double biotinylation technique to track age related changes in forward scatter and CD147 expression on erythrocytes. Forward scatter is a measure of the size of the cells and an age related decline in size of erythrocytes has been suggested (Waugh et al., 1992). We found a biphasic decline in forward scatter of biotin^{low} population of erythrocytes. These results indicate that a significant fall in forward scatter occurs after about 35 days of erythrocyte age (Fig. 5). Earlier fall before 20 days of age could perhaps be related to the maturation of reticulocytes into erythrocytes. As reticulocytes shed vesicles to mature into erythrocytes, a reduction in size is expected (Johnstone et al., 1987; Waugh et al., 1997).

Uniform and constant fall in CD147 expression occurred as erythrocytes aged in circulation. Interestingly, the fall in CD147 expression was steepest in the first ten days of aging of biotin^{low} population of erythrocytes, a phase when the loss of this population had not yet started. It is possible that the relatively faster loss of CD147 during early stages could also be related to the shedding of vesicles from erythrocytes, though this possibility needs further examination. CD147 marker is involved with recirculation of erythrocytes passing through spleen (Coste et al., 2001). Decline in CD147 expression on aging erythrocytes could therefore be a factor in the removal of erythrocytes by preventing their recirculation.

A large number of factors may determine the survival of erythrocytes in circulation. Some of these factors may be age dependent and others not. The double biotinylation technique described in this paper can be utilized to directly study age related changes in age defined populations of erythrocytes. This method may not work in humans due to the presence of naturally occurring anti-biotin antibodies in human blood (Dale et al., 1994). However it can work in dogs and rabbits where biotinylation per se does not influence the survival of circulating erythrocytes (Suzuki and Dale, 1987; Christian et al., 1996; Hoffmann-Fezer et al., 1997). Furthermore, the technique may be used to isolate age defined populations of erythrocytes by flow cytometric cell sorting, and facilitate gaining of further insight into the mechanisms of erythrocyte turnover in blood.

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