Aging and destruction of blood erythrocytes in mice

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Erythrocytes evolved to carry out the crucial function of transportation of oxygen from lungs to various tissues in the body and to carry back carbon dioxide. Absence of a nucleus in erythrocytes facilitates their easy movement in blood capillaries and make space for large quantities of haemoglobin, the oxygen carrier molecule, but renders the cells ill equipped to effectively repair themselves from oxidative and other damages. Turnover rate of erythrocytes is fast and the average life span of murine erythrocytes in blood is only 50 days. What age related changes occur in circulating erythrocytes and which of these changes are crucial determining factors in triggering the destruction of old erythrocytes, are not well understood. The main reason for a lack of information in this area is the fact that there were not available any good and easy to perform technique to identify circulating erythrocytes of different age groups. We have recently developed a technique that involved *in vivo* biotinylation of erythrocytes in two steps that has allowed us for the first time to objectively assess age-related changes in blood erythrocytes. We have utilized this technique to study various aspects of aging of murine erythrocytes. On the basis of these results, our current thinking about the process of erythrocyte aging *in vivo* is summarized in the this article.

Keywords: Aging, erythrocytes, oxidative damage, phosphatidyl serine, RBC turnover.

ERYTHROCYTES constitute almost 99.9% of all the blood cells (excluding platelets). Erythrocytes are terminally differentiated cells lacking nucleus, and cannot undergo cell division. As the main function of erythrocytes is to remove carbon dioxide and supply oxygen to different organs, the cells shuttle between various organs and the lungs, where carbon dioxide bound to haemoglobin in erythrocytes is replaced with oxygen. A reversal of the process occurs in peripheral organs where oxygen is released by the haemoglobin and carbon dioxide absorbed. These repetitive cycles of oxidation and reduction result in significant oxidative stress in erythrocytes. Erythrocytes are, thus subject to significant oxidative stress. In addition, erythrocytes traverse through skin where they may be exposed to damaging penetrating radiations or through areas with ongoing inflammatory responses, where they may be exposed to damaging mediators. As the cell repair machinery is highly deficient in erythrocytes, erythrocytes damaged due to oxidative or other forms of stress are unable to undertake self-repair and are prone to be destroyed. For this reason, erythrocyte turnover rate is high and 1% of all the circulating erythrocytes in human blood (2% in rodents) are destroyed each day[1–4]. The mechanism of destruction of erythrocytes involves macrophages in the reticulo-endothelial system that recognize the erythrocytes that must be removed, and eliminate them through the process of phagocytosis[5–7]. Evidence for an alternative mechanism of erythrocyte destruction through cell lysis has also been published[8–10].

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Models of erythrocyte turnover

Theoretically, two extreme models may be conceived for the destruction of circulating erythrocytes. These models can be termed (i) critical age model, and (ii) random destruction model. The first model suggests that the destruction of erythrocytes depends solely upon their age. Thus, erythrocytes may be destroyed when they reach a particular critical age and consequently have accumulated sufficient cellular damage. If this model is correct, all members of a cohort of erythrocytes that enter the blood at a given time, would survive till they reach a particular critical age and then be destroyed rapidly as shown in Figure 1 *a*. In the random destruction model, erythrocytes in circulation may be killed at random irrespective of their age. In this case, members of a cohort of erythrocytes freshly entering the blood would start dying from the very beginning and the expected survival kinetics of the erythrocyte cohort could be as depicted in Figure 1 *b*. If both processes are partially responsible for the destruction of erythrocytes, the survival kinetics may lie somewhere in between the two curves shown in Figure 1. In any case, the destruction of erythrocytes in blood should be finely balanced with the entry of new erythrocytes in blood circulation, so that at any given time the number of erythrocytes in circulation remains constant.

The problem we have been pursuing in our laboratory is of determining the actual survival kinetics of erythrocytes and correlating it with the two models discussed

above. Understanding of these issues is important not only for gaining an insight into the mechanisms of normal turnover of circulating erythrocytes but also the changes that occur in the blood counts of erythrocytes in various forms of anaemia and polycythemias.

There is a considerable amount of information in the literature about the changes that take place in erythrocytes upon aging[11–13]. However, most of this data can be criticized since there are no good methods available for fractionating the circulating erythrocytes into populations of different age groups. Most popular method of physically separating the erythrocytes is based upon the buoyant density of erythrocytes. It is generally believed that the buoyant density of erythrocytes increases as they age[14]. There is, however, no clear proof of a direct relationship between buoyant density and age of the circulating erythrocytes, and the assumption of such a relationship has been questioned[15,16]. In an old study by Allison and Burn[17], blood group O bearing erythrocytes were infused in a host with blood group A and at subsequent time points, group O bearing erythrocytes were isolated by removing the host erythrocytes through agglutination by anti-group A antibodies, in order to study changes in aging group O erythrocytes. Unfortunately, this technique suffered from many technical problems. Ganzoni *et al.*[18] and later Mueller *et al.*[19] used another method involving sequential hyper-transfusing of blood cells in rats that allowed aging erythrocytes to be enriched. Besides being cumbersome and animal intensive, this technique also resulted only in relative enrichment of old erythrocytes and did not yield a pure preparation of old erythrocytes. Moreover, none of these techniques allowed a comparison of erythrocytes of different age groups within a given sample of erythrocytes.

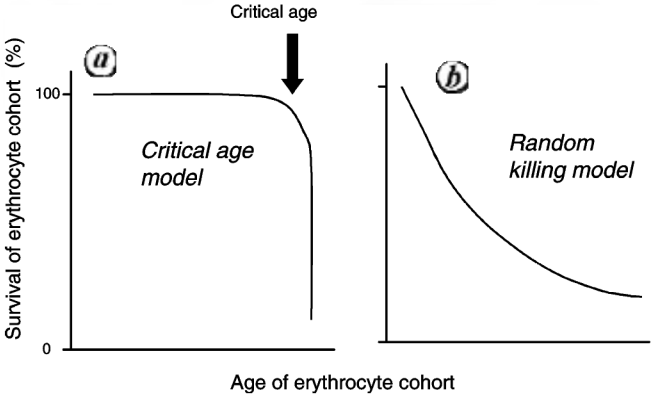

Figure 1. Two extreme models of erythrocyte destruction. If age is the sole criterion for earmarking erythrocytes for killing, members of a cohort of erythrocytes generated at a given time point would survive *in toto* and keep circulating till a critical age is reached. Thereafter, accumulated damage to senescent cells may cross a critical mark for their recognition and destruction by macrophages ***a***. If erythrocytes are destroyed randomly irrespective of their age, a given cohort of erythrocyte population would start dwindling as soon as it is released fresh in blood circulation ***b***.

A breakthrough in the technology for studying age-related changes in blood erythrocytes came in 1987, when Suzuki and Dale demonstrated that erythrocytes could be labelled with biotin and the biotin label remains unchanged if the cells are transfused back in mice[20,21]. Hoffmann-Fezer *et al.*[22,23] 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. In these techniques, biotinylated erythrocytes could be stained *ex vivo* with avidin coupled to a fluorochrome and analysed by flow cytometry. After labelling all circulating erythrocytes *in vivo*, if mice were bled after few days, the fresh erythrocytes released in circulation were biotin negative and very young erythrocytes could thus be distinguished from the rest by flow cytometry. Similarly, if such mice were bled after about 50 days of biotinylation step, only a small fraction of very old biotinylated erythrocytes survived in circulation that could either be analysed flow cytometrically or even isolated by using avidin affinity beads[18]. Although this elegant technique could be used to study very young or very old erythrocytes, it could not be used to isolate intermediate age groups of erythrocytes or for direct comparison of very young and very old erythrocytes from the same mouse. Recently, we have been able to further modify the single step in *in vivo* biotinylation technique of Hoffmann-Fezer *et al.*[22,23] by introducing a second *in vivo* biotinylation step[24,25]. Our modified technique allows for the first time, a systematic study of changes in cohorts of erythrocytes, from the time they are first released in circulation till they age and fade away from circulation. The technique can also be used to simultaneously study very young and very old erythrocytes from the same mouse[24–27].

The double *in vivo* biotinylation technique for tracking age-related changes in circulating erythrocytes

The principle of double *in vivo* biotinylation (DIB) technique has been explained in Figure 2. In the first biotinylation step comprising three daily intra-venous injections of BXN (Biotin-X-NHS Ester, 1 mg/mouse/day), all erythrocytes become labelled with biotin. After resting the mouse for 5 days, about 10% of the blood erythrocytes are biotin negative, these being the cells that were released in blood circulation after the completion of the first biotinylation step. At this stage, a single injection of 0.6 mg BXN labels the freshly released erythrocytes and render them biotin^{low} as compared to older biotin^{high} erythrocytes that were labelled in the first biotinylation step. Any time thereafter, blood erythrocytes isolated from a DIB labelled mouse may be categorized in three groups, viz. a biotin^{high} (oldest age) group, a biotin^{low}

(intermediate age) group and a biotin-negative (youngest age) group. All three groups of erythrocytes can be identified and enumerated by staining with strapavidin coupled to a fluorochrome like FITC or APC, followed by flow cytometric analysis. It should be noted that instead of a 5 days gap, the second step of biotinylation could as well be done after a 2, 3 or 4 days gap. These altered gaps would result in a decrease in the proportion of the biotin^{low} erythrocyte band (i.e. 4, 6 and 8% respectively instead of 10% for 5 days gap), without any effect on the actual pattern of age dependent changes on erythrocytes.

Figure 3 shows the flow cytometric patterns of biotin labelling on erythrocytes isolated after different steps in the DIB technique. By gating on any category of erythrocytes and using a second or a third staining antibody, it is possible to compare the expression of various markers of interest on the oldest, intermediate and the youngest erythrocyte populations. Furthermore, as the DIB labelled mouse ages, the biotin^{low} cohort of erythrocytes gradually moves from very young to very old part of the spectrum. By analysing test bleeds from DIB labelled mice taken at various time points, it is possible to track age-related changes taking place in the biotin^{low} cohort of erythrocytes in blood.

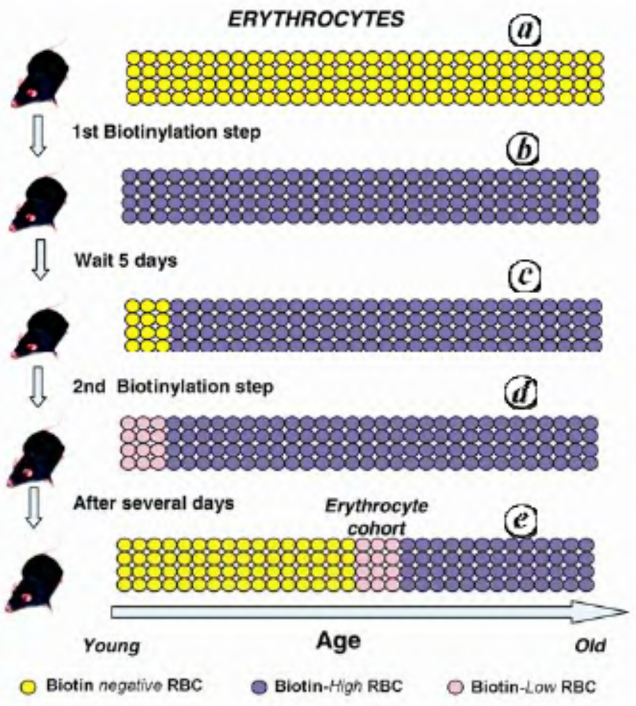

Figure 2. Double *in vivo* biotinylation (DIB) technique for tracking age-related changes on circulating erythrocytes. ***a***, Normal circulating erythrocytes are biotin negative. ***b***, After three daily injections of BXN (biotin-X-NHS Ester 1 mg/mouse/day) all erythrocytes become biotin labelled. ***c***, After 5 days, about 10% erythrocytes that were produced fresh after the first biotinylation step are biotin negative. ***d***, A second *in vivo* biotinylation step with a single injection of 0.6 mg BXN results in relatively lower intensity of biotin labelling on freshly produced band of erythrocytes. ***e***, At all later time points, biotin-high population represents the oldest erythrocyte population in circulation, biotin-low population represents a cohort of erythrocytes produced during a limited time window (5 days in this example) between first and second biotinylation steps. Biotin-negative population represents the youngest cells produced after the second biotinylation step. Erythrocytes can be stained with strapavidin-FITC/APC *ex vivo* and analysed on flow cytometer as shown in Figure 3. For details of methodology see references 24 and 25.

Kinetics of erythrocyte destruction in blood circulation in mice

Survival kinetics of cohorts of blood erythrocytes expected from the two extreme models of erythrocyte destruction has been discussed above. DIB technique allowed us to investigate the actual kinetics of loss of erythrocytes generated and released in blood during a short and defined window of time (an erythrocyte cohort). For this purpose, mice were DIB labelled and sacrificed at different time points when blood samples were taken and the percentage survival of the biotin^{low} cohort of erythrocytes determined. Combined results of survival kinetics data obtained on 14 mice have been presented in Figure 4. Scrutiny of whole survival data plotted against time indicated that up to 10 days of age, survival of erythrocyte cohorts remained around 100%. Thereafter, a decline in survival was observed. Mean rate of decline in survival was about 1.3% per day till 40 days of age and doubled thereafter to 2.8% per day. The three phases denoted in Figure 4 have essentially been demarcated based upon the best visual fit of the data points. Mean slopes of the survival curve in the three phases and their standard deviations are also shown in Figure 4. Correlation coefficients remained above 0.9 for phases 2 and 3 of the survival curve. These results indicate that a steady killing of erythrocyte cohort starts as early as 10 days after their release in the blood and the rate of killing becomes greater as the cohort after they are 40 days old. By day 60, almost all erythrocytes of the biotin^{low} cohort vanish from the blood. This data agrees well with the accepted life span of erythrocytes in mouse blood[1,2].

Age-related changes in CD47, CD147 and phosphatidyl serine expression on erythrocytes

We have tracked three important markers (CD47, CD147 and phosphatidyl serine (PS)) on erythrocytes that are important in regulation of cell survival. CD47 is a marker on erythrocytes that protects erythrocytes from phagocytosis by macrophages[28–30]. CD47 molecules on erythrocytes interact with their specific receptors on macrophages and send an inhibitory signal that suppresses the phagocytic response. CD147 expression on erythrocytes facilitates their recirculation from spleen to blood[31]. Decreased CD147 marker results in trapping of erythrocytes in spleen. Figure 5 shows the kinetics of age-related changes in the expression of CD47 and CD147 markers on erythro-

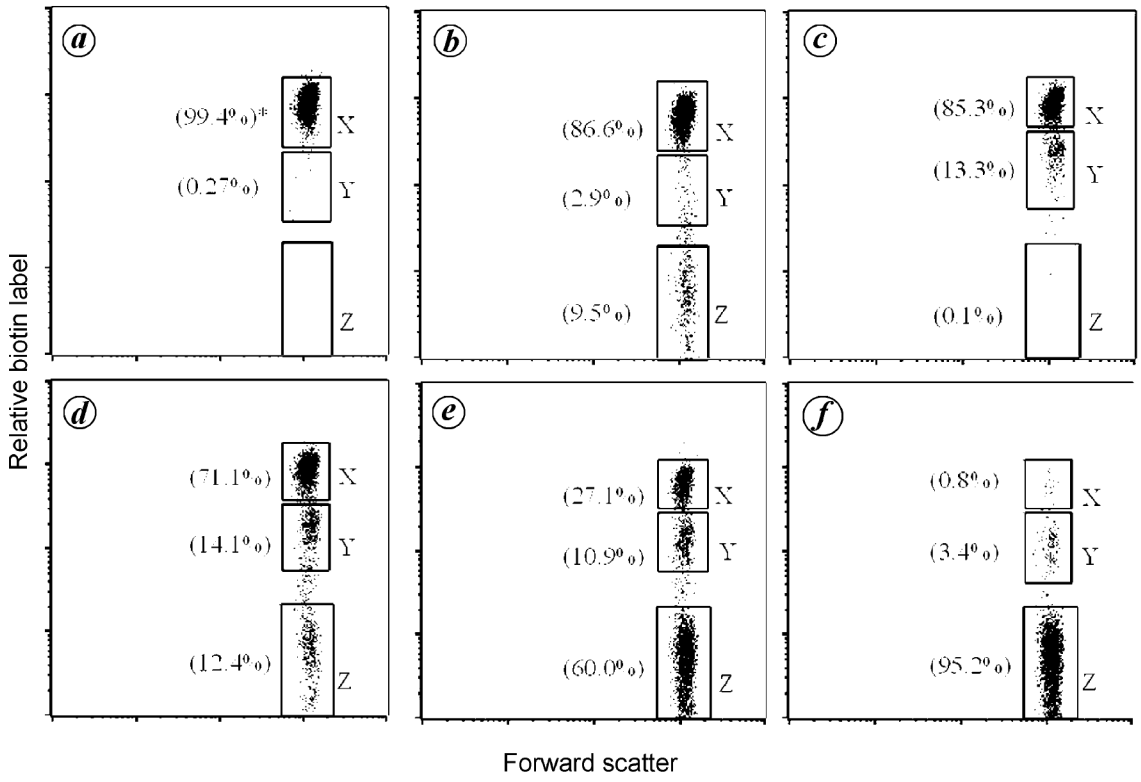

Figure 3. Demarcation of three discrete erythrocyte populations in blood using a DIB procedure. C57Bl/6 mice were administered intravenously three daily doses of 1 mg BXN (first biotinylation step). After a five day rest, a single additional dose of 0.6 mg BXN was administered (second biotinylation step). 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[24,25]. 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-negative populations of erythrocytes. Values in parentheses represent percentage of cells in different boxes.

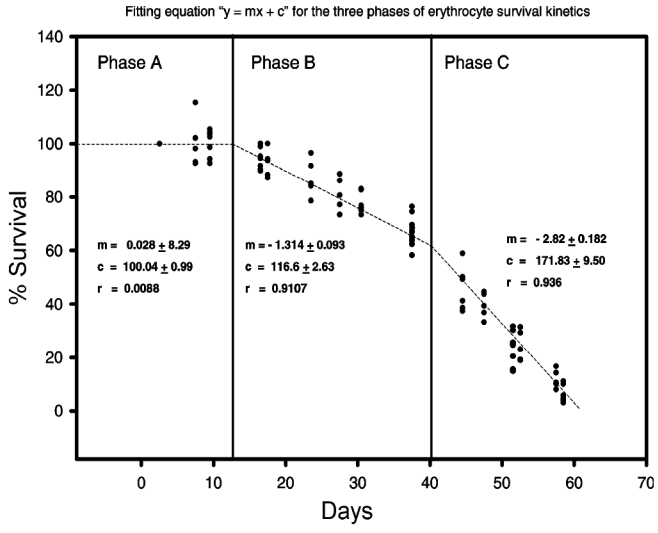

Figure 4. Composite data on the decay of a defined erythrocyte cohort in circulation. C57Bl/6 mice were DIB labelled and sacrificed at different time points. At different time points, biotin^{low} population (defined in Figures 2 and 3) were assessed as percentage of all circulating erythrocytes. Combined data of survival kinetics from 14 mice show the decay of the biotin^{low} cohort of erythrocytes as a function of age. Correlation coefficients and slopes of the three phases of the decay curve have been calculated.

cytes cohort. These results show that both markers decline significantly with age on circulating erythrocytes. Lower density of CD47 and CD147 markers would render the aging erythrocyte populations susceptible to phagocytosis and promote their entrapment in spleen. Both these mechanisms may be responsible for the gradual loss of erythrocytes from circulation.

PS externalization is an early marker for cells undergoing apoptosis[32]. While erythrocytes lack nucleus and may not generate a classical apoptotic response, a process like apoptosis termed eryptosis has been described for erythrocytes that also involves PS externalization[33,34]. It has been suggested that PS externalization may be responsible for the destruction of senescent erythrocytes[34–36]. Using DIB technique, we monitored changes in PS expression on erythrocytes as a function of age. Figure 6 shows that PS expression on unfractionated erythrocytes remains low (<1%). When biotin^{low} cohort was monitored for PS expression, no significant age-related increase was found in this parameter. Very young erythrocytes had a small percentage of PS positive cells (about 4%), but at later time points PS expression remained very low (Figure 6). We could not find an age-related increase in PS expres-

sion on erythrocytes and our results therefore do not support a role of PS externalization in the destruction of old erythrocytes in circulation. It is possible that we did not see the enhanced PS expression because such cells were immediately removed from circulation by macrophages. To test this possibility, we depleted mice of macrophages (90% macrophage depletion) but did not find elevation of PS and erythrocytes in macrophage-depleted mice also[27]. Some other workers have demonstrated a significant increase in PS expression in old erythrocytes in mice by using annexin V-FITC reagent to assess PS expression. We have recently found that old murine erythrocytes have elevated green autofluorescence with emission spectrum similar to that of FITC[26]. Annexin V-FITC may therefore not be an appropriate reagent for comparing PS expression in young and old erythrocytes since the autofluorescence in old erythrocytes would introduce artefacts resulting in erroneous results. We used Annexin V-APC instead of annexin V-FITC to stain erythrocytes for PS expression. Emission spectrum of APC does not overlap with that of the green autofluorescence of old erythrocytes and we could avoid the artefact associated with using FITC labelled annexin V. Using annexin V-APC, we found no evidence for elevated PS expression on old erythrocytes (Figure 6).

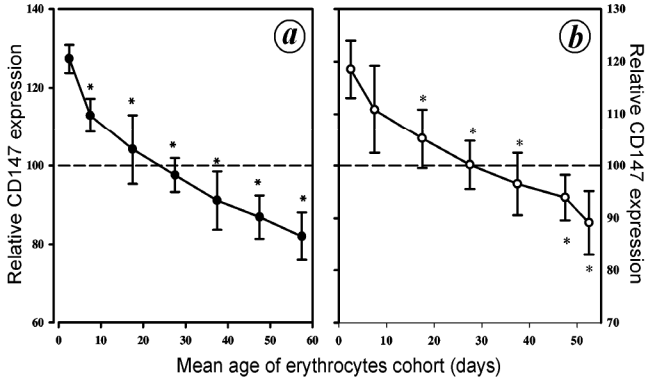

Figure 5. Kinetics of decline in CD147 and CD47 marker expression on erythrocyte cohort as a function of age. Mouse erythrocytes were labelled with DIB technique as described in Figures 2 and 3. CD147 (***a***) and CD47 (***b***) expression on biotin^{low} cohort of erythrocytes was examined 5, 15, 25, 35, 45 and 50 days after the second biotin dose (panel ***b***). At each time point erythrocytes were stained with anti-CD147 and anti-CD47 mabs and examined on flow cytometer. At each time point, mean channel of CD147 and CD47 expression was assessed for all erythrocyte and for biotin^{low} cohort of cells. Mean channel values for biotin^{low} cohort as percentage of mean channel for all erythrocytes have been plotted. Each point on the graph represents mean ± SD of observations on five mice. *$P < 0.005$ for comparison of different time points with the first time point. Details of methodology are available elsewhere[24,26].

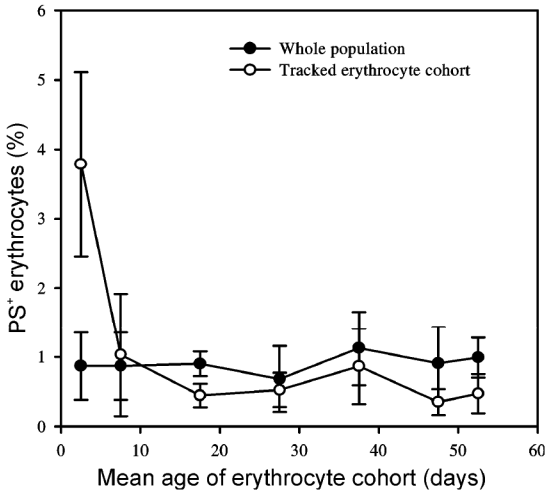

Figure 6. Kinetics of age dependent phosphatidylserine (PS) externalization on circulating erythrocytes. Mice were labelled by DIB technique as described in Figure 3 and sacrificed at different time points. Erythrocytes samples were stained with strapavidin-FITC and Annexin V-APC, and analysed on a flow cytometer. PS expression was studied on biotin^{low} (open circles) and whole erythrocyte (closed circles) populations at all time points. Each point in the graph represents mean ± SD of data from 3 to 5 mice.

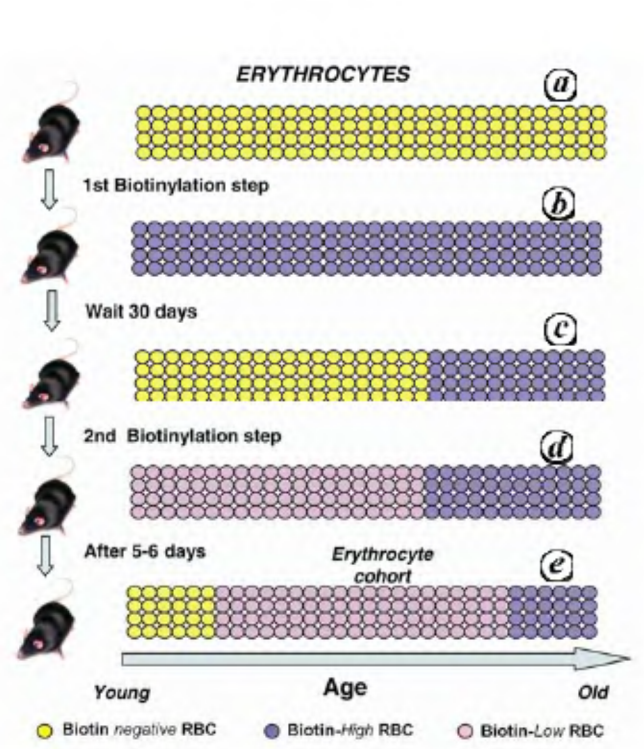

Figure 7. DIB technique for simultaneous identification and study of youngest and oldest erythrocytes in blood circulation. ***a***, Normal circulating erythrocytes are biotin negative. ***b***, After three daily injections of BXN 1 mg/mouse/day all erythrocytes become biotin labelled. ***c***, After 30 days, about 60% erythrocytes that were produced fresh after the first biotinylation step are biotin-negative. ***d***, A second *in vivo* biotinylation step with a single injection of 0.6 mg BXN results in relatively lower intensity of biotin labelling on freshly produced band of erythrocytes. ***e***, After 5–6 days, biotin high population represents the oldest erythrocyte population in circulation, whereas the biotin-negative population represents the youngest cells produced after the second biotinylation step. Biotin-low population represents a wide spectrum of erythrocytes of intermediate age group. Erythrocytes can be stained with strapavidin-FITC/APC *ex vivo* and analysed on flow cytometer as shown in Figure 3. For details of methodology see references 24 and 25.

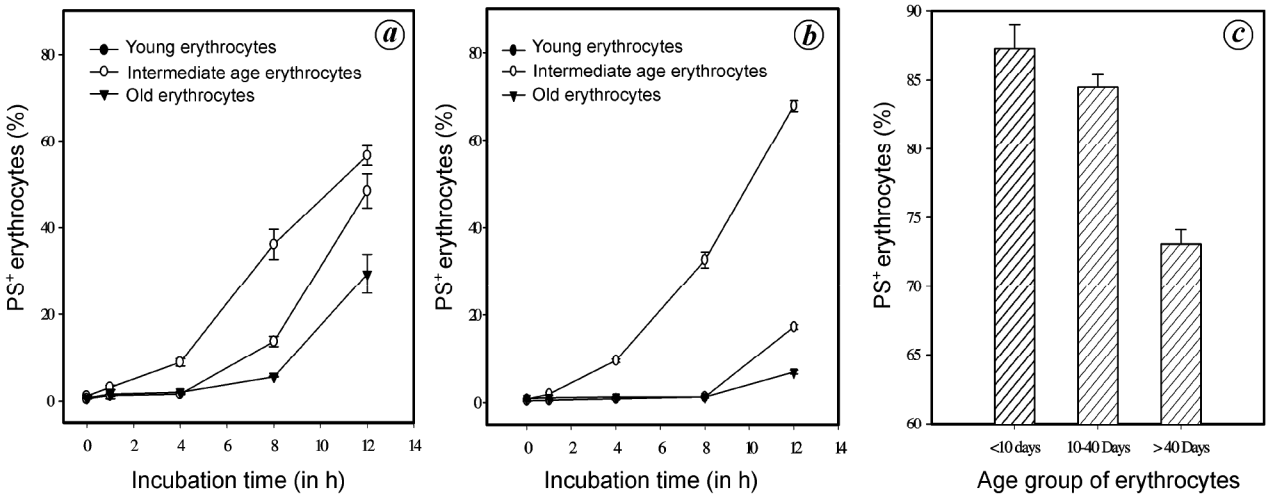

Figure 8. Effect of various stresses on PS externalization on different age groups of erythrocytes. Mouse erythrocytes were biotin labelled by the DIB technique as described in Figure 7. This biotinylation strategy enabled identification of erythrocytes belonging to young (<10 days), intermediate (10–40 days) and old (>40 days) age groups. Labelled erythrocytes were collected and incubated for 12 h in HEPES buffered saline with ***a***, 5 mM 2-DOG or ***b***, 1 mM $CaCl_2$ or ***c***, 1 h with 1 mM $CaCl_2$ and 0.5 μM calcium ionophore. At different time points, panels A and B, percentage of PS^+ erythrocytes was examined after staining with SAv-APC and annexin V-FITC. Each point represents mean ± SD of three observations. In panel ***a***, PS expression on intermediate as well as old erythrocytes was significantly different from young group ($P < 0.05$ at all time points except 0 h). Intermediate and old groups were significantly different at 8 and 12 h time points ($P < 0.05$). In panel ***b*** also, PS expression on intermediate as well as old erythrocytes was significantly different from young group ($P < 0.05$ at all time points except 0 h). Intermediate and old groups were significantly different at 12 h time points ($P < 0.05$). In panel ***c***, PS expression is significantly lower in old group ($P < 0.01$) as compared to young and intermediate groups. Young and intermediate group values are not significantly different.

Stress and erythrocyte turnover

Exposure of mouse erythrocytes to a variety of stress inducing reagents results in high degree of PS externalization[37–39]. We could confirm these results but found marked differences in the PS externalization in young and old erythrocytes, exposed to stress-inducing reagents. In order to compare the PS expression on stress exposed youngest and the oldest populations of erythrocytes from the same mouse, we developed a fresh strategy of the two-step biotinylation protocol that has been explained in Figure 7. By using this strategy of biotinylation, the youngest and the oldest populations of blood erythrocytes could be identified and examined simultaneously for stress-induced PS externalization. Figure 8 indicates that exposure to deoxy glucose (DOG), $CaCl_2$ and $CaCl_2$ + calcium ionophore (reagents known to induce stress in erythrocytes), induced high levels of PS expression in young erythrocytes but relatively low levels on erythrocytes of intermediate ages. No increase in PS expression was seen on old erythrocytes exposed to stress. Furthermore, when DIB labelled erythrocytes were exposed to stress *in vitro* and then infused back into mice, only young population of infused erythrocytes was rapidly and selectively eliminated from the blood (Figure 9). These results suggest that stress-induced PS externalization is more prominent in young erythrocytes. We therefore hypothesize that young erythrocytes in blood circulation, exposed to stress may be eliminated through eryptosis or through phagocytosis by effectors macrophages recognizing enhanced PS expression on erythrocytes.

Current hypothesis about erythrocyte turnover in blood

Due to lack of appropriate techniques, an objective assessment of erythrocyte turnover in mice was not available. A variety of techniques involving the infusion of tagged erythrocytes have been utilized to assess the life span of erythrocytes *in vivo*. Since the infused erythrocytes were unfractionated and represented a mixture of erythrocytes of all ages, precise determination of rate of erythrocyte destruction was not possible. Using the new DIB technique, we have been able to follow the survival kinetics of a cohort of erythrocytes of defined age, with a degree of precision not possible so far. The actual survival kinetics of an erythrocyte cohort determined experimentally by using the DIB technique appears to fall somewhere in between the patterns predicted by the two extreme models given in Figure 1. The fact that the numbers of erythrocytes within the age defined cohort started to fall as early as 10 days after the cells entered the circulation, clearly indicates that the erythrocyte destruction is not solely determined by the age of the cohort and random killing must at least partially account for the loss of erythrocytes from circulation. Furthermore, the fact that the rate of decline in cell numbers is lower in the beginning (10–40 days) and increases in the later part of the life span of erythrocytes, (>40 days) suggests that the cell destruction may also be partially determined by age-related changes that occur on circulating erythrocytes. The factors that make circulating erythrocytes susceptible to random (age independent) killing are not known. How-

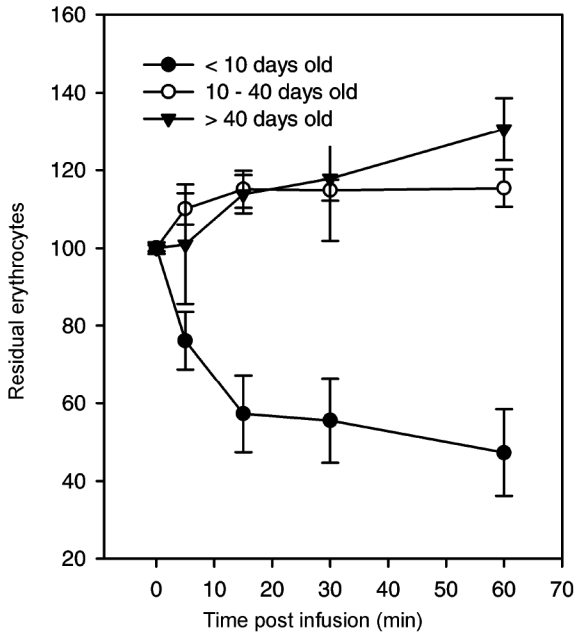

Figure 9. Clearance of different age group of PS^+ erythrocytes from the circulation. Mouse erythrocytes were labelled using DIB technique as described in Figure 7. Erythrocytes were collected and stained with a green fluorescent dye CFSE and incubated for 8 h in the HEPES buffer saline with 1 mM $CaCl_2$ (stress inducing agent). After *in vitro* treatment, these erythrocytes were infused in fresh mice and blood samples were collected at different time points. Erythrocytes were stained with SAv-APC and annexin V–PE and analysed flow cytometrically. Residual infused erythrocytes ($CFSE^+$ population) were grated and proportions of different age group of infused erythrocytes and the numbers of residual PS^+ erythrocytes in each age group were determined. Values of percentage of residual PS positive erythrocyte in different age group of erythrocyte population at different time point have been shown. Each point in the graph represents the mean ± SD of values obtained in three separate experiments. As compared to the first time point, the decline in the numbers of infused young PS^+ erythrocytes in circulation was statistically significant (range $P < 0.05$–0.001).

ever, it is tempting to speculate that younger erythrocytes that get damaged due to oxidative or other forms of stress may extrude PS and be removed through phagocytosis. Since PS extrusion is more efficient in younger erythrocytes, it is likely that this mechanism of stress-induced random killing may be relatively more prevalent in younger populations of erythrocytes.

We have demonstrated that the PS extrusion response to stress is significantly lower in senescent erythrocytes. Older damaged erythrocytes may therefore neither externalize PS nor be eliminated by the random killing route. We have also demonstrated a steady fall in CD47 and CD147 expression during aging of erythrocyte cohorts. Factors like a fall in CD47 and CD147 expression (rather than PS extrusion) may render older erythrocytes susceptible to phagocytosis by macrophages. It is possible that the combined effects of these changes may result in a greater rate of destruction of erythrocytes above 40 days of age. That would explain an increase in the slope of the survival curve after 40 days time point.

Conclusion

We have developed a new double *in vivo* biotinylation (DIB) technique that allows objective assessment of age-related changes that occur in circulating blood erythrocytes. Using this technique, we have been able to determine the precise survival of age-defined cohorts of erythrocytes in mouse blood. Further, on the basis of our results of survival kinetics and changes in the expression of survival determining markers like CD47, CD147 and PS, we have suggested the factors that may determine the shape of the survival curve of erythrocytes in mouse blood.

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