Age-dependent increase in green autofluorescence of blood erythrocytes

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Green auto-fluorescence (GAF) of different age groups of mouse blood erythrocytes was determined by using a double *in vivo* biotinylation (DIB) technique that enables delineation of circulating erythrocytes of different age groups. A significant increase in GAF was seen for erythrocytes of old age group (age in circulation >40 days) as compared to young erythrocytes (age <15 days). Erythrocytes are removed from blood circulation by macrophages in the reticulo-endothelial system and depletion of macrophages results in an increased proportion of aged erythrocytes in the blood. When mice were depleted of macrophages for 7 days by administration of clodronate loaded liposomes, the overall GAF of erythrocytes increased significantly and this increase could be ascribed to an increase in GAF of the oldest population of erythrocytes. Using the DIB technique, the GAF of a cohort of blood erythrocyte generated during a 5 day window was tracked *in vivo*. GAF of the defined cohort of erythrocytes remained low till 40 days of age in circulation and then increased steeply till the end of the life span of erythrocytes. Taken together our results provide evidence for an age dependent increase in the GAF of blood erythrocytes that is accentuated by depletion of macrophages. Kinetics of changes in GAF of circulating erythrocytes with age has also been defined.

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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. Erythrocytes traverse the body through circulation and can be subjected to a variety of stresses including oxidative stress that might result from the rough and tumble of passing through capillaries, exposure to penetrating radiations in skin and from minor and major immune reactions occurring at different locations with in the body. Since erythrocytes lack nucleus and protein synthetic machinery, their capacity to repair damage resulting from stresses is limited. Protective enzymes against oxidative damage are present in erythrocytes but activity of these enzymes decrease in the old age erythrocytes. (Clark 1988; Deiss 1999). In senescent erythrocytes therefore, oxidative damage accumulates (Clark 1988; Deiss 1999; Arese *et al* 2005).

The production of fluorescent products has long been known to accompany the peroxidation process. The interaction of malonyldialdehyde and the amino groups of phospholipids and proteins during lipid peroxidation results in the formation of fluorescent chromolipids (Bidlack and Tappel 1973). Rahman *et al* (1995) showed an induction of autofluorescence in human erythrocytes after the treatment with hydrogen peroxide. Cheng *et al* (1999) demonstrated that humic acid induced oxidative damage resulted in autofluorescence in erythrocytes. Autofluorescence of erythrocyte has also been used to assess lipid peroxidation in erythrocytes from thalassemic (Senok *et al* 1998) and uremic patients (Stoya *et al* 2002). Spontaneous glycation of glycated proteins and carbohydrates may also generate autofluorescence in erythrocytes (Vigneshwaran *et al* 2005).

Keywords. Biotin; green autofluorescence; *in vivo* biotinylation; macrophage depletion; murine erythrocyte aging

Abbreviations used: BXN, biotin-X-NHS Ester; DIB, double *in vivo* biotinylation; DMF, dimethylformamide; GAF, green auto fluoresence; PBS, phosphate buffered saline

Since oxidative stress may accumulate in erythrocytes and some agents that induce oxidative stress have been shown to induce auto-fluorescence, we hypothesized that old erythrocytes in blood circulation may acquire higher autofluorescence. This hypothesis can be tested by isolating old and young erythrocytes and examining their autofluorescence. This has however not been easy since no good technique was available for demarcating erythrocytes of different age groups in blood. It is believed that erythrocytes become denser and acquire higher buoyant density as they age (Rennie *et al* 1979; Bennett and Kay 1981). This proposition has however not been conclusively proved and it is not clear how much overlap is there amongst the densities of erythrocytes of different age groups (Morrison *et al* 1983; Clark 1988; Dale and Norenberg 1990). Recently we have developed a double *in vivo* biotinylation (DIB) technique to clearly demarcate the erythrocytes in different age groups (Khandelwal and Saxena 2006; Khandelwal *et al* 2007). This technique also enables us to tag a cohort of fresh erythrocytes released in blood during a defined time window and track changes in this cohort of cells as they age in blood circulation (Khandelwal and Saxena 2006, Khandelwal *et al* 2007). In the present study we have used the DIB technique to compare the autofluorescence of erythrocytes of different age groups of erythrocytes as also the kinetics of the change of autofluorescence in aging mouse erythrocytes. Our results provide evidence for a distinct increase in the autofluorescence in aged erythrocytes that is accentuated when macrophages responsible for removing aged erythrocytes from circulation, are depleted *in vivo*.

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^{o}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 (BXN) was procured from Calbiochem (La Jolla, CA, USA), Streptavidin Allophycocyanin (SAv APC) was from BD biosciences. (San Diego, CA, USA). Fetal Bovine serum was obtained from Hyclone (South Logan, Utah, USA). Dimethylformamide (DMF) and other reagents were from Sigma-Aldrich (India). Clodronate containing liposomes were made by the procedure described elsewhere (Van Rooijen and Van Kesteren-Hendrikx 2003).

2.3 *Biotin labelling*

Double *in vivo* biotinylation (DIB) of circulating erythrocytes was done as described before (Khandelwal and Saxena 2006). Briefly, mice were given three daily intravenous injections of 1 mg of BXN dissolved in 20 μl of DMF and 250 μl of phosphate buffered saline (PBS). This step resulted in high intensity biotin labelling of all erythrocytes in blood. 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 or 25 days after the last injection of the first step biotinylation. This step resulted in a relatively lower intensity labelling of biotin on erythrocytes released in blood after the first biotinylation step. Erythrocytes produced after the second injection of BXN remained unlabelled.

2.4 *Macrophage depletion*

Mice were depleted of macrophages by the intravenous injections of clodronate loaded liposomes as described earlier (Khandelwal *et al* 2007). Briefly mice were given repeated intravenous injections of clodronate containing liposomes (10 ml / kg) at 4 day intervals. This treatment resulted in a >80% decline in macrophage population as assessed by the expression of the macrophage marker F4/80 (Khandelwal 2007).

2.5 *Flow cytometry*

Blood was collected in PBS containing EDTA (5 mM) and washed 3 times with ice cold normal saline containing 10 mM HEPES buffer (pH 7.4) and 1% FBS. For flow cytometric studies, 1 million cells were stained with streptavidin allophycocyanin by the procedure recommended by the manufactures. After adding the SAv APC erythrocytes were incubated at room temperature for 30 min and washed. Stained erythrocytes were immediately analysed on FACScalibur (with blue and red lasers) 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.

2.6 *Statistical analysis*

Statistical analysis was done using Sigma plot software. Data are presented as mean $\pm$ SD. Significant values were calculated using student's *t*-test.

3. Results

3.1 *Green autofluorescence of blood erythrocytes of different age groups*

Oxidative stress is known to enhance the autofluorescence of erythrocytes (Bidlack and Tappel 1973; Rahman *et al* 1995). Since aged populations of blood erythrocytes may have been exposed to a longer duration of oxidative stress as compared to the younger populations of erythrocytes, we hypothesized that old erythrocyte populations may have greater autofluorescence. We have recently developed a DIB technique that permits simultaneous analysis of different age groups of erythrocytes in blood circulation (Khandelwal and Saxena 2006; Khandelwal *et al* 2007). Using this technique we examined the autofluorescence of mouse erythrocytes of different age groups. DIB technique demarcates these populations based upon the extent of biotinylation of the cells. Figure 1A shows this demarcation where biotin^{high} (box X, age group >40 days), biotin^{low} (box Y, age group 15-40 days) and biotin negative (box Z, age group <15 days) populations of erythrocytes have been defined. Green auto fluorescence (GAF) of erythrocytes gated in boxes X, Y and Z are shown in figure 1B, and indicate that the mean autofluorescence intensities increased from 2.76 for erythrocytes of age < 15 days, to 4.37 for erythrocytes of age > 40 days. Results in figure 1A, B are for a representative experiment. Data from 7 such experiments is summarized in figure 1C and clearly shows that the GAF of erythrocytes is significantly greater in older populations of blood erythrocytes.

3.2 *Enhanced green autofluorescence in erythrocytes from macrophage depleted mice*

It is believed that aged populations of erythrocytes are removed by phagocytosis by macrophages in the reticuloendothelial system (Bennett and Kay 1981; Deiss 1999; Kiefer and Snyder 2000). Therefore, if macrophages are depleted *in vivo* in mice, old populations would survive for longer time and the proportion of aged erythrocytes would increase in blood circulation. Using clodronate liposomes to deplete macrophages *in vivo* we have recently demonstrated the accumulation of old erythrocytes in blood (Khandelwal *et al* 2007). Since older erythrocytes have greater GAF, erythrocytes from macrophage depleted mice may show a further increase in GAF. This proposition was examined by measuring the GAF of blood erythrocytes from control and macrophage depleted mice. Results in

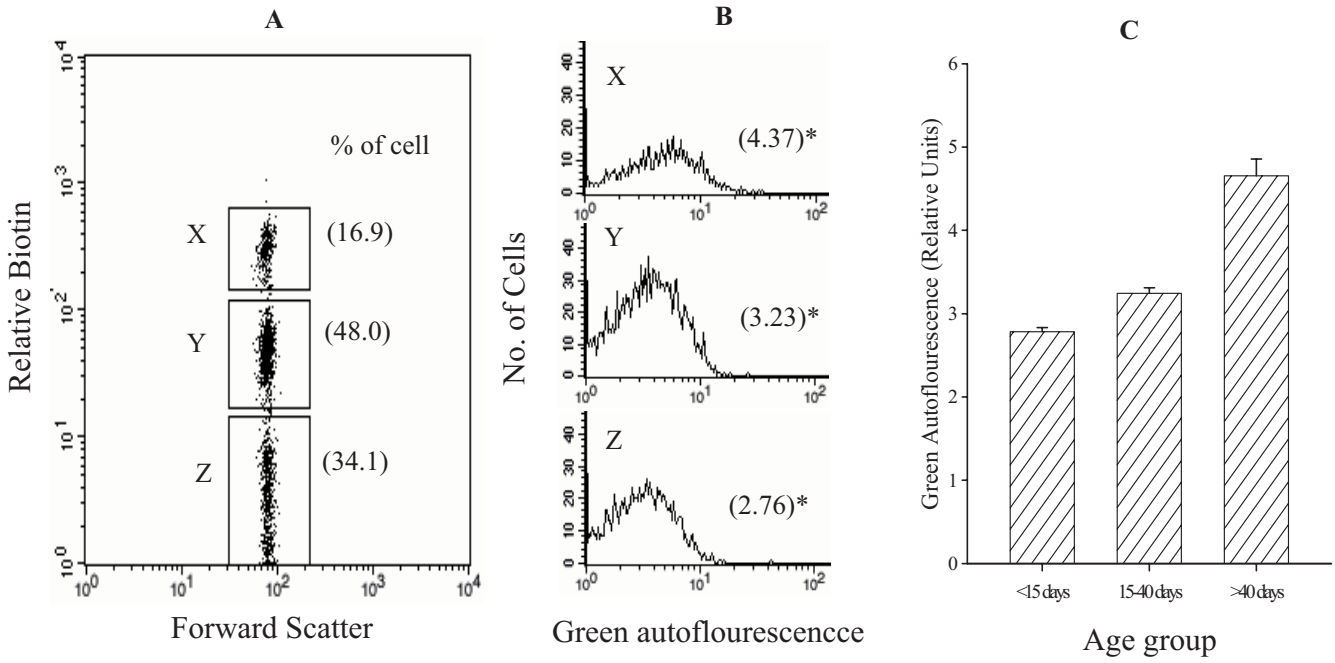

Figure 1. GAF of erythrocytes belonging to young, intermediate and old age groups. Circulating erythrocytes were labelled by DIB technique where the second step of low intensity biotinylation was performed, 25 days after the initial step high intensity biotinylation. After 15 days of the second biotinylation step, erythrocytes were stained with SAv-APC. **(A)** Demarcation of the three erythrocyte populations i.e. the old (> 40 days of age, box X), intermediate (age 15 to 40 days, box Y) and young (age <15 days, box Z) erythroyctes. **(B)** Flow cytometric histograms of the GAF emitted by the X, Y and Z boxed populations of erythrocytes. Values in parentheses denote the mean fluorescence intensities (MFIs). **(C)** Mean and SD of the MFI values obtained from 7 mice. * *P<0.001* for difference from youngest age group.

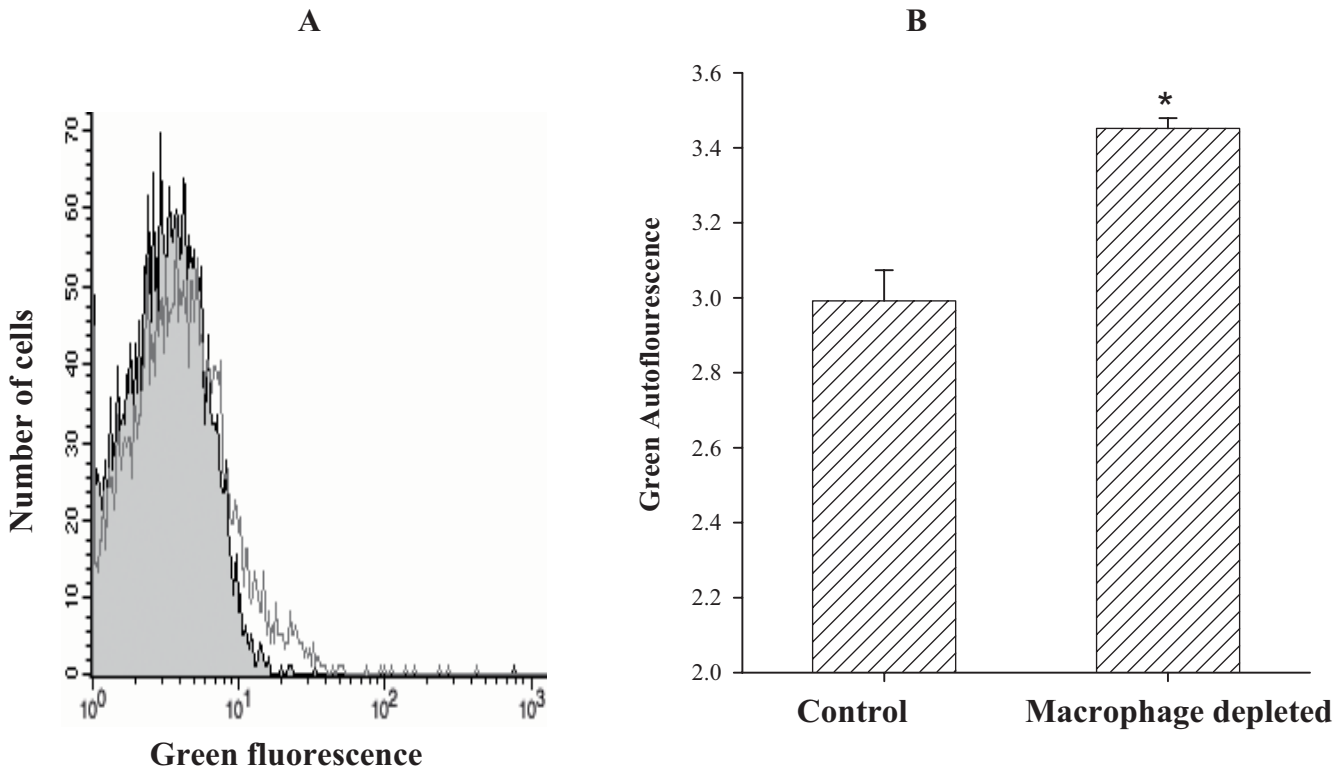

Figure 2. GAF of the erythrocytes from the normal and macrophage depleted mice. Mice were kept macrophage depleted for 7 days by the repeated intravenous injections of clodronate liposomes. GAF of unstained whole populations of erythrocytes from control (filled histogram) and macrophage depleted (open histogram) mice was determined by flow cytometry (**A**). (**B**) The mean ± SD of mean fluorescence intensity values from 6 mice. Significance of difference: $*P < 0.001$. The three bars in figure 1B represent variable fractions of the cell populations and a simple mean cannot be taken. A weighted mean must be taken and if that is done, the values of mean fluorescence in figure 2B and 1C are quite comparable.

figure 2A show that the GAF distribution of erythrocytes from macrophage depleted mice was shifted slightly to right, indicating a small increase in autofluorescence. The increase in mean GAF was around 14% and was statistically significant (figure 2B). Using the DIB technique, GAF of erythrocytes of different age groups from control and macrophage depleted mice were compared. Results in figure 3 show that the GAF of young and intermediate age groups of erythrocytes from control and macrophage depleted mice did not differ significantly. However in the >40 days age group, average autofluorescence was about 30% greater in mice from macrophage depleted mice ($P < 0.001$). These results show that accumulation of aged erythrocytes in blood is accompanied with a concomitant increase in GAF of blood erythrocytes. Moreover, this increase is seen only in the oldest compartment of blood erythrocytes.

3.3 *Kinetics of increase in GAF of erythrocytes with age*

DIB technique also permits us to tag a cohort of erythrocytes produced within a defined window of time (biotin^{low} erythrocytes) and then to track changes on this population as it ages in blood. Using this technique we studied the kinetics of age dependent increase in GAF of erythrocytes. Results in figure 4 track the age dependent increase in GAF of biotin low population of erythrocytes that were produced within a window of 5 days, and compare their GAF with the GAF of unfractionated blood erythrocytes at each time point. These results clearly show that up to 40 days of age, GAF of whole blood erythrocytes and the demarcated cohort of erythrocytes were comparable. From 50 day time point onwards, a relatively steep increase in GAF of aging erythrocytes was observed. At the last time point, the GAF of the aging cohort of erythrocytes was about 80% greater than the mean GAF of unfractionated erythrocytes.

4. Discussion

Aim of the present study was to test the hypothesis that aging of erythrocytes in blood circulation is accompanied with an enhanced GAF. In the first set of experiment, we found that the GAF was significantly greater (by about

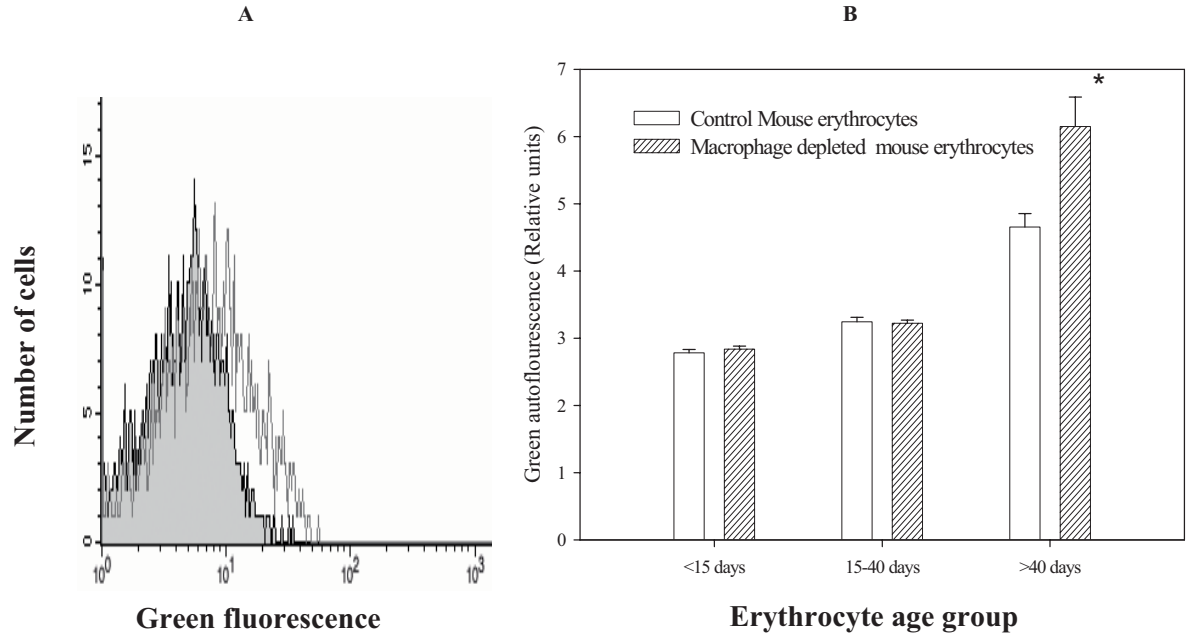

Figure 3. GAF of the different age groups of erythrocytes in the normal and macrophage depleted mice. Blood erythrocytes of control and macrophage depleted mice were biotinylated by DIB technique as explained in legend to figure 1 and GAF of the three age groups of erythrocytes were determined. Left panel shows the GAF distributions of the >40 days old erythrocytes from the control (filled histogram) and macrophage depleted (empty histogram) mice. The comparison of MFI of GAF in different age group of erythrocytes in the control and macrophage depleted mice has been shown in right panel. Each bar represents mean ± SD of MFIs of HAF from 7 control and 7 macrophage depleted mice. *$P < 0.001$ for difference between erythrocytes from control and macrophage depleted mice.

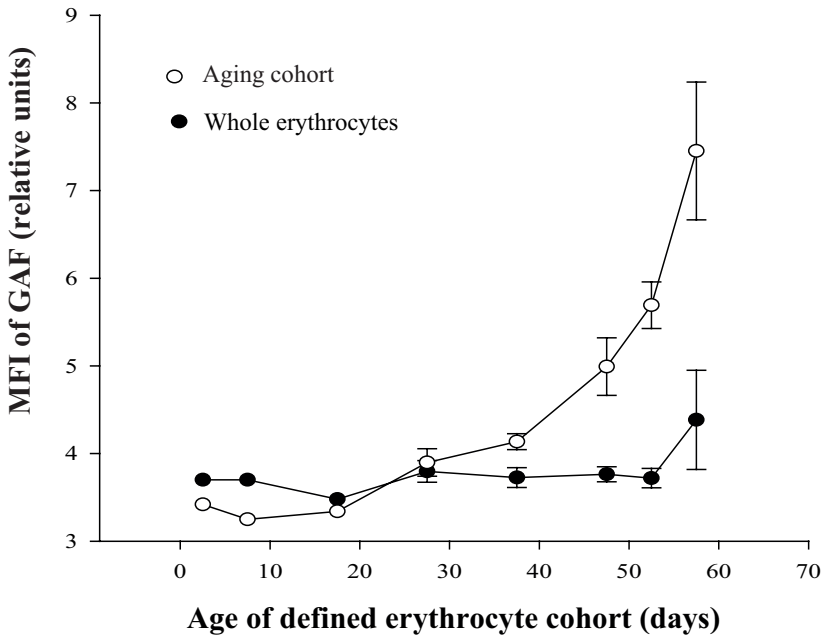

Figure 4. Kinetics of age dependent changes in the GAF of blood erythrocytes. Erythrocytes were biotinylated by DIB technique to enable tracking of a erythrocyte cohort population generated during five days between the first and the second steps of biotinylation. At different time points after the second step of biotinylation, blood erythrocytes were stained with SAv-APC and the erythrocyte cohort was gated as biotin^{low} population (as box Y in figure 1). Mean fluorescence intensities of the green autofluorescence of whole erythrocyte population (filled dots) and the biotin^{low} cohort of erythrocytes (open dots), were determined. Each point represents mean ± SD of observations on 5 mice.

55%) on erythrocytes above 40 days of age as compared to erythrocytes less than 15 days of age. The DIB technique we utilized to specifically gate erythrocytes of different age groups has been explained in details elsewhere (Khandelwal and Saxena 2006; Khandelwal *et al* 2007). The gating of erythrocyte populations by this technique is unequivocal since different age groups of erythrocytes are not distinguished by some inherent cellular property that changes with age. Using an inherent property like buoyant density as a marker of aging has the disadvantage that if there are changes in such properties with age, the changes are gradual and small. Moreover there are generally considerable overlaps between ranges of such parameters amongst different age groups of erythrocytes, which make it difficult to clearly and unequivocally define erythrocyte populations of different ages. DIB technique involves labelling different age groups of erythrocytes with clear cut ranges of biotin and erythrocytes can thereafter be gated flow cytometrically after staining with fluorescence tagged avidin. Results in figure 1A illustrate this point where boxes X, Y and Z denote old, intermediate and young populations of erythrocytes respectively. It should be noted that we have used avidin probe coupled with APC fluorochrome with emission range (640 to 680 nm light) distinct from green fluorescence (emission 515 to 545 nm light), and thus avoided overlap between the windows used for gating the cells and measuring their GAF.

Macrophages in the reticuloendothelial system are primarily responsible for removing erythrocytes from blood circulation (Bennett and Kay 1981; Deiss 1999; Kiefer and Snyder 2000). Administration of clodronate loaded liposomes specifically depletes macrophages in mice and other experimental animals (Van Rooijen and Sanders 1994; Van Rooijen and Van Kesteren-Hendrikx 2003). If old erythrocytes with greater GAF are not eliminated and survive longer in the blood, an increase in overall GAF of blood erythrocytes is expected in mice depleted of macrophages. Our results have shown a significant increase in GAF of erythrocytes from macrophage depleted mice and have further demonstrated that the increase is due to enhanced GAF of old erythrocytes. Kinetics of changes in autofluorescence in an aging cohort of blood erythrocytes indicated that significant increase in GAF of aging cells is first seen in erythrocytes above 45 days of age and further goes up relatively steeply till the end of the erythrocyte life span (figure 4).

Besides demonstrating the autofluorescence in old populations of erythrocytes, our results are also important for another reason. Many studies utilize fluorescent probes like FITC coupled antibodies to study age related changes in expression of specific antigens on blood erythrocytes, e.g. phosphatidylserine expression in old erythrocytes (Singer *et al* 1986; Bratosin *et al* 1995, 1998; Boas *et al* 1998; Fossati-Jimack *et al* 2002; Manodori and Kuypers 2002). Since the background gate in flow cytometric studies is set by using whole blood erythrocytes, higher autofluorescence of old erythrocytes is interpreted as an increase in the expression of antigen under study. Our present results show that such increases may primarily or at least partially be due to higher autofluorescence of the old erythrocytes and necessitate a re-look at the inferences drawn in such previous studies. Our study thus underlines the importance of using old erythrocytes for setting the background gate in flow cytometric studies of markers on old erythrocytes using probes like FITC whose emission sepecrum overlaps with the autofluorescence of old erythrocytes.

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