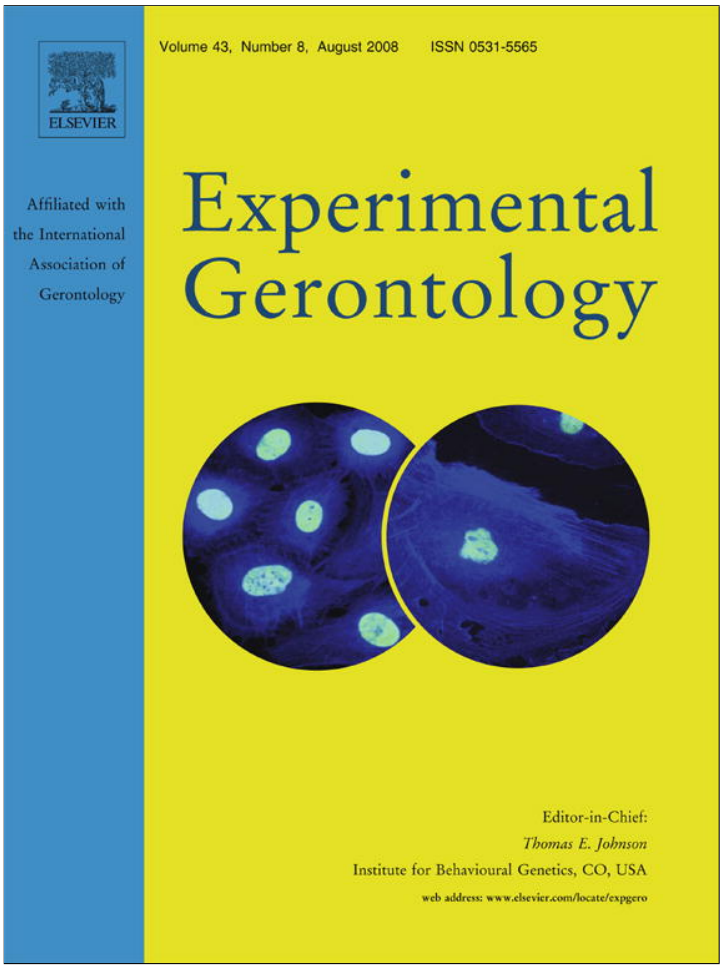

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A role of phosphatidylserine externalization in clearance of erythrocytes exposed to stress but not in eliminating aging populations of erythrocyte in mice

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ABSTRACT

Age dependent changes in phosphatidylserine (PS) externalization were studied in mouse erythrocytes of different age groups (range 1–55 days) by using a newly developed double *in vivo* biotinylation (DIB) technique. Around 3–4% of the erythrocytes freshly released in the circulation were PS^+ but this proportion fell rapidly to 1% or less and did not increase at later time points. Blocking erythrocyte clearance from the circulation by *in vivo* depletion of macrophages (by treatment with clodronate loaded liposomes) for up to 7 days did not result in accumulation of PS^+ erythrocytes in the circulation indicating that the low percentage of PS^+ cells within old erythrocytes (age >40 days) was not related to the clearance of PS^+ erythrocytes by macrophages. *In vitro* treatment with stress inducing agents like deoxyglucose or Ca^{++}/calcium ionophore resulted in a marked induction of PS externalization in mouse erythrocytes and this effect was most prominent in the youngest erythrocyte population (age <10 days). Kinetics of clearance of different age groups of stress exposed erythrocytes after intravenous infusion into recipient mice indicated that the young erythrocytes were cleared at fastest rate from the circulation as compared to erythrocytes of older age groups. Within young erythrocytes exposed to stress, PS^+ erythrocytes were preferentially cleared. Taken together our results suggest that PS externalization is unlikely to have a role in the removal of old erythrocytes from blood circulation but may have a role in the clearance of stressed and damaged young erythrocytes in 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). Altered features of circulating erythrocyte that earmark them for destruction are, 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).

Apoptosis is a well known mechanism for the clearance of the nucleated cell but its role in the clearance of erythrocytes from the circulation is not well understood. Changes similar to apoptosis like the extrusion of phosphatidylserine in erythrocyte membrane have been shown to occur in aged populations of erythrocytes (Bosman et al., 2005). Increased intracellular calcium ion concentration causes PS externalization in the erythrocytes that is mediated by the calcium dependent phospholipid scramblase (Zhou et al., 1997). Klarl et al. (2006) demonstrated that the removal of extracellular glucose led to depletion of cellular ATP, stimulated PKC activity, enhanced serine phosphorylation of membrane proteins, decreased cell volume, and increased PS externalization. Erythrocytes with PS on their outer surface are recognized by the macrophage and cleared from the circulation (Tanaka and Schroit, 1983; Schroit et al., 1985; McEvoy et al., 1986). PS externalization has also been suggested as a mechanism for elimination of aged erythrocytes from blood circulation (Connor et al., 1994; Bratosin et al., 1998; Boas et al., 1998; Manodori and Kuypers, 2002).

Recently we have developed a double *in vivo* biotinylation (DIB) technique to unambiguously identify blood erythrocyte populations of defined age groups (Khandelwal and Saxena, 2006). This technique 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 study PS externalization in

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erythrocyte populations of different age groups and their clearance from blood. Our results indicated that no significant increase occurred in the percentage of PS^+ erythrocytes in the aging erythrocyte population. Exposure to in vitro stress, however, resulted in rapid PS externalization in young erythrocytes and this PS^+ population of erythrocytes was rapidly cleared from blood circulation. Removal of PS^+ erythrocytes may therefore play an important role in clearance of stress damaged erythrocytes from blood but may not have a role in the removal of aging population of erythrocytes.

2. Materials and methods

2.1. Mice

Inbred C57BL/6 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.

2.2. Reagents

Biotin-X-NHS Ester (BXN) was from Calbiochem (La Jolla, CA). Streptavidin Fluorescein-Isothiocyanate (SAv FITC), Streptavidin Allophycocyanin (SAv-APC) and Annexin V FITC/PE/APC were from BD biosciences (San Diego, CA). SAv PE was from Caltag laboratories, USA. Fetal Bovine serum was obtained from Hyclone (South Logan, Utah). 5-(And-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Molecular Probes, Eugene, OR. Dimethylformamide (DMF), calcium ionophore A23187, bovine serum albumin, 2-deoxyglucose (DOG) and other reagents were from Sigma–Aldrich (India). Clodronate containing liposomes were made by the procedure described elsewhere (Van Rooijen et al., 1990; Van Rooijen and Van Kesteren-Hendrikx, 2003).

2.3. Determination of Phosphatidylserine exposing erythrocytes

Phosphatidylserine expressing erythrocytes were analyzed flow cytometrically after staining with fluorochrome conjugated Annexin V. Fresh blood erythrocytes or erythrocytes after various treatments were washed with HEPES buffered saline (HBS, 10 mM HEPES in 165 mM NaCl solution, pH adjusted to 7.4 using 1 N NaOH solution) and resuspended in the HBS containing 2.5 mM calcium chloride. One million cells were taken and incubated with Annexin V FITC/APC for 20 min at room temperature, washed and resuspended in the HBS with 2.5 mM $CaCl_2$ and analyzed on flow cytometer immediately. In absence of Annexin V staining negligible fraction of biotinylated erythrocytes (0.091% ± 0.042, mean ± SD of seven observations) was false positive for PS expression. Biotinylation of erythrocytes had no effect on their level of PS expression.

2.4. Macrophage depletion

Mice were depleted of macrophages by i.v. injections of clodronate loaded liposomes as described earlier (Khandelwal et al., 2007). Briefly mice were given intravenous injections of clodronate containing liposomes (10 ml/kg body weight) on day 1, 4 and 7 and sacrificed on day 8. Control mice received PBS alone.

2.5. Double in vivo biotin labeling

Double *in vivo* biotinylation (DIB) of circulating erythrocytes was done as described before. (Khandelwal and Saxena, 2006). Briefly, mice were given three daily i.v. injections of 1 mg of biotin-X-NHS Ester (BXN) dissolved in 20 μl of dimethylformamide (DMF) and 250 μl of phosphate buffered saline (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 or 25 or 30 days after the last injection of the first step biotinylation.

2.6. In vitro treatment of erythrocytes

A suspension of 2% erythrocytes was made in 10 mM HEPES buffered saline and incubated in the presence of various reagents [deoxyglucose (DOG) (5 mM), calcium chloride (1 mM), calcium ionophore A23187 (0.5 μM) in the 96 well U-bottom culture plate for different times at 37 °C and 5% CO_2. After harvesting the erythrocytes were washed three times with HBS and stained with SAv FITC and Annexin V APC.

2.7. Study of in vivo clearance of erythrocytes

Mouse blood (500 μl) was collected in anticoagulant Heparin (10 U) and mixed with a solution of 1.3 mg biotin X NHS (BXN) dissolved in 26 μl DMF and 4 ml of PBS and incubated at 37 °C for 30 min with gentle shaking. After incubation erythrocytes were washed three times with PBS containing 1% FBS. A 16% (v/v) suspension of biotinylated erythrocytes was prepared in the HBS with 500 μM $CaCl_2$ and incubated for 3 min at 37 °C. Calcium ionophore (A23187) was added to a final concentration of 1 μM and cells were further incubated for 60 min. After treatment erythrocytes were washed once with HBS containing 2.5 mM EDTA (to remove calcium) and thrice with HEPES buffered saline containing 1% bovine serum albumin (BSA, to remove the ionophore). Subsequently erythrocytes were washed twice with plain HEPES buffered saline, resuspended in HBS and injected in the mouse through tail vein. Blood samples were collected from the mice after 5, 15, 30, 60 and 120 min. Erythrocytes were washed and stained with SAv PE and Annexin V FITC in the HEPES buffer saline containing 2.5 mM $CaCl_2$.

For studying the clearance of erythrocytes of different age groups, DIB labeled mouse erythrocytes were prepared as described above, collected and stained *in vitro* with the CFSE {5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester} dye. For this purpose a 5% (v/v) suspension of DIB labeled erythrocytes in PBS was incubated with CFSE (final concentration 10 μM) for 15 min at 37 °C. Erythrocytes were washed twice with PBS containing 1% FBS, once with plain HBS and suspended at 2% v/v concentration in HBS. DIB and CFSE labeled erythrocytes were incubated in 6-well plates in the presence of 1 mM $CaCl_2$ for 8 h at 37 °C/5% CO_2. After incubation erythrocytes were harvested and washed three times with HBS, resuspended in HBS and injected in the mouse through tail vein. Blood samples were collected at different time points from infusion, as above from the tail vein. Erythrocytes were washed with HBS and stained with SAv-APC and Annexin V-PE. $CFSE^+$ cells represented residual infused erythrocyte population. Gated $CFSE^+$ cells could be further analyzed flow cytometrically for surviving subpopulations of erythrocytes of different ages through SAv-APC stain and for PS externalized erythrocytes by Annexin V-PE stain.

2.8. Flow cytometry

Stained erythrocytes were analyzed immediately without fixing on FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using Cell Quest pro software for acquisition and analysis. A minimum of 10,000 events were recorded for each sample.

2.9. Statistical analysis

Statistical analysis was done using Sigma plot software. Data are presented as means ± SD/SEM. Significant values were calculated using Student's *t*-test.

3. Results

3.1. Phosphatidylserine positive (PS^+) erythrocyte population in different age group of erythrocytes

We have recently developed a new double *in vivo* biotinylation (DIB) technique to objectively identify circulating erythrocytes of defined age groups (Khandelwal and Saxena, 2006). This technique was used to assess the expression of PS on the outer membrane of young (age <15 days), intermediate (age 15–40 days) and old (age >40 days) mouse erythrocytes. For this purpose, biotin-X-NHS ester, a biotinylation agent, was administered in mice as per the scheme described in Section 2 so that after SAv FITC staining, the blood erythrocytes could be delineated into biotin high (age >40 days), biotin low (age 15–40 days) and biotin negative (age <15 days) populations. Erythrocyte preparations were double stained with annexin V APC to reveal PS externalization in these subpopulations of erythrocytes. Results of a representative experiment in Fig. 1 show that the young, intermediate and old erythrocytes comprised 17.2%, 48.8% and 32.5% of the total erythrocyte population, respectively (Fig. 1A). Percentage of PS positive erythrocytes in these groups was 0.47%, 0.19% and 0.41%, respectively (Fig. 1B). Pooled results from seven such experiments (Fig. 1C) indicate that the percentage of PS^+ erythrocytes in young, intermediate and old age erythrocytes was 0.44 ± 0.19%, 0.37 ± 0.19% and 0.46 ± 0.09%, respectively. These results show that there was no significant difference in the percentage of PS^+ erythrocytes in erythrocyte populations of different age groups.

3.2. Survival kinetics and age dependent phosphatidylserine externalization on circulating mouse erythrocytes

DIB technique can be used to tag a population of erythrocytes freshly released in the blood stream over a defined window of time and track changes on this selected population as it gradually ages in circulation (Khandelwal and Saxena, 2006). By using this technique, we tracked the kinetics of age dependent changes in PS externalization in mouse erythrocytes. Results in Fig. 2 show that the 3–4% of the erythrocytes freshly released in the circulation (day 1 time point) were PS^+ but this proportion fell rapidly to 1% or less at day 6 or later time points. Even as the proportion of selected erythrocyte cohort fell progressively within whole blood erythrocytes, the percentage of PS^+ erythrocytes within this cohort remained unaltered and did not increase with age (Fig. 2). These results clearly indicate that the aging of erythrocytes in blood circulation is not associated with an increased PS externalization.

3.3. Effect of macrophage depletion on the numbers of PS^+ erythrocytes in blood circulation

Results so far indicated that there was no apparent age dependent increase in PS^+ erythrocytes in mouse blood circulation. These results however do not rule out the possibility that the aged erythrocytes might have had enhanced PS externalization but PS^+ cells were rapidly removed from the circulation, thereby preventing their accumulation in blood. Major pathway for erythrocyte destruction involves phagocytosis by macrophages in the reticulo-endothelial system and we have previously shown that *in vivo* depletion of macrophages results in accumulation of old erythrocytes in blood (Khandelwal et al., 2007). Effect of macrophage depletion was therefore examined on the levels of PS^+ erythrocytes in blood. Results in Table 1 show that the proportion of PS^+ erythrocytes remained unchanged even in the macrophage depleted mice. These results do not support an increased externalization of PS on aging populations of blood erythrocytes.

3.4. Stress induced PS externalization in erythrocytes

Exposure to different types of stress as well as induced by increase in intracellular calcium ion concentration are known to induce PS externalization in erythrocytes. (Allan and Thomas, 1981; Bucki et al., 1998; Bratosin et al., 2001; Klarl et al., 2006). Even though we found no age related increase in PS externalization in erythrocytes, we further investigated if age of erythrocytes was

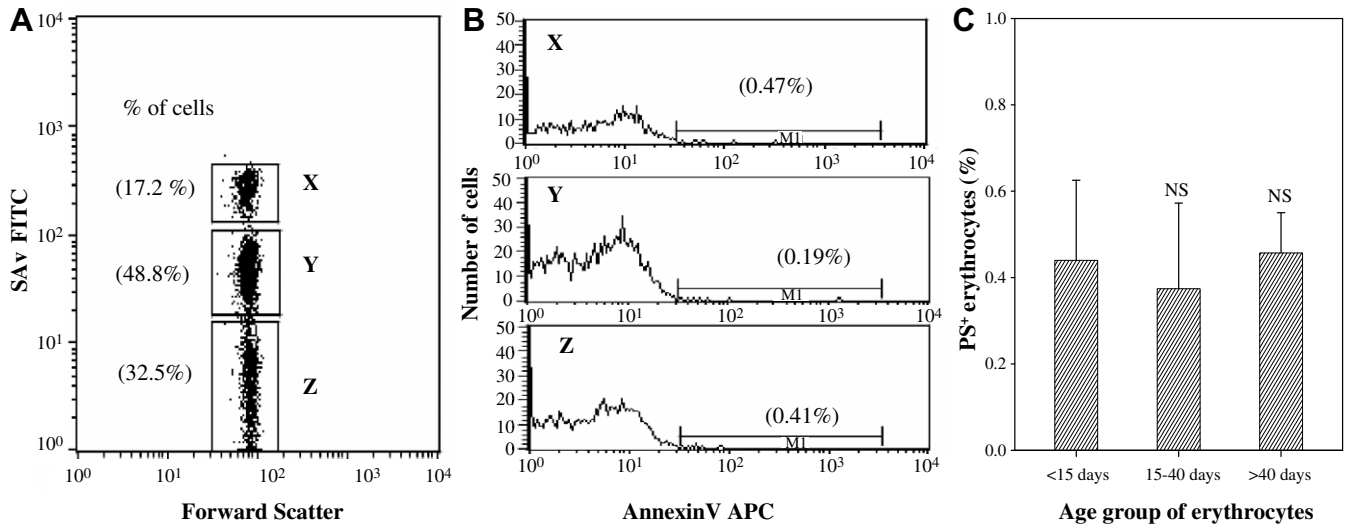

Fig. 1. Phosphatidylserine positive (PS^+) erythrocyte population in different age group of erythrocytes. Circulating erythrocytes were labeled by DIB technique where by mice were administered 1 mg BXN each on three consecutive days, followed by a 25 days rest and then a single dose of 0.6 mg BXN as described in Section 2. After 15 days of the second biotinylation step, erythrocytes were collected and stained with SAv FITC and Annexin V APC. (A) Demarcation of the three erythrocyte populations i.e. the old (>40 days of age, box X), intermediate (age 15–40 days, box Y) and young (age <15 days, box Z) erythrocytes. (B) Flow cytometric histograms showing the PS^+ erythrocytes in the X, Y and Z boxed populations of erythrocytes. Values in parentheses denote the % of PS^+ erythrocytes. (C) Summary of results obtained from seven mice. Each bar represents mean ± SD (NS, not significant).

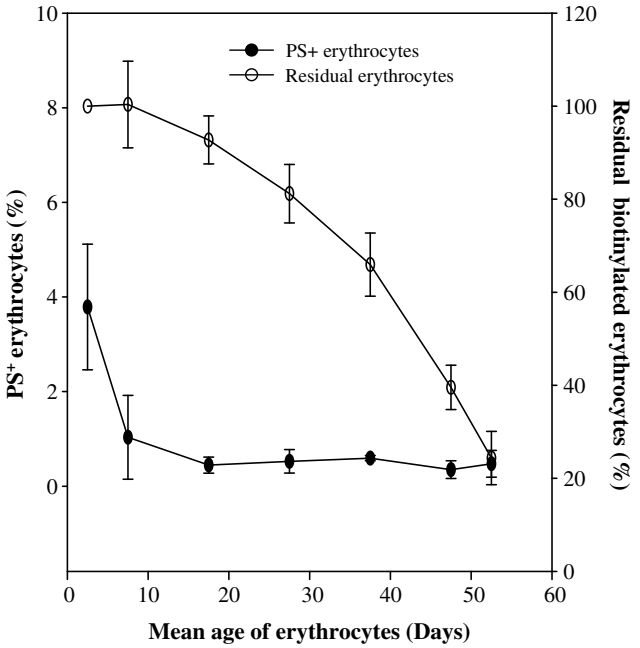

Fig. 2. Survival kinetics and age dependent phosphatidylserine externalization on circulating mouse erythrocytes. Mouse erythrocytes was labeled by DIB technique where the second step of low intensity biotinylation was performed 5 days after the initial step of high intensity biotinylation as described in Section 2. Survival and PS expression was studied on biotin^{low} population during its life span in blood. Biotin^{low} erythrocyte population was delineated by SAv FITC staining and PS expression on this subpopulation was determined by staining with annexin V APC. Each point in the graph represents mean ± SD of data from five mice. As compared to the first time point the decline in PS expression on all subsequent time points was statistically significant (range $p < 0.05$–0.001).

a factor in stress induced PS externalization. To examine this possibility, DIB labeled erythrocyte populations were subjected to *in vitro* stress and PS externalization studied in different age groups of erythrocytes. DIB labeled erythrocytes were incubated in buffered saline containing 5 mM deoxyglucose (DOG), or 1 mM $CaCl_2$, or 1 mM $CaCl_2$ along with 0.5 μM calcium ionophore A23187. At different time points, erythrocytes were stained with SAv-APC and annexin V FITC and analyzed on a flow cytometer. Results in Fig. 3 show that in all cases, young erythrocytes (age <10 days) were most prone to PS externalization in response to stress whereas the old erythrocytes (age >40 days) were the least susceptible in this regard. Incubation of erythrocytes in HEPES buffered saline alone, in absence of any stress inducing agent induced significant PS externalization in young erythrocytes (e.g. 14.9 ± 0.25% PS$^+$ cells after 12 h of incubation) that was nonetheless markedly lower than the response seen in presence of the stress inducing agents. Treatment with stress inducing agents per se had no significant effect on the level of biotinylation of erythrocytes (results not shown).

3.5. Clearance of infused PS exposing erythrocytes from the circulation

PS externalization is an early indicator of apoptosis in nucleated cells, and PS$^+$ cells are rapidly cleared by macrophage phagocytosis (Fadok et al., 1992; Schlegel and Williamson, 2001; Kagan et al., 2003). We hypothesized that PS exposing erythrocytes could similarly be susceptible to phagocytosis by macrophages. In order to test this hypothesis we studied the kinetics of clearance of PS$^+$ and PS$^-$ erythrocytes in mice. Whole erythrocyte preparations were labeled with biotin *in vitro* and PS externalization was induced on these cells by treatment with calcium and calcium ionophore A23187. These cell preparations were then infused intravenously in mice and blood samples were collected at different time points to assess the survival of infused erythrocytes. An analysis of the biotin labeled erythrocytes recovered from mice indicated that PS$^+$ erythrocytes were cleared at a much faster rate as compared to PS$^-$ erythrocytes (Fig. 4). Within two hours of infusion, almost all the PS$^+$ erythrocytes were removed from the circulation whereas 60–80% of PS$^-$ erythrocytes survived (Fig. 4).

3.6. Survival of different age groups of control and stressed erythrocytes from blood circulation

Results so far show that (a) there is no increase in PS externalization in old erythrocyte populations, (b) stress induced PS externalization was significantly more in young as compared to the old erythrocytes, and (c) PS exposing erythrocytes were cleared efficiently from the blood stream. Taken together, our results suggest that PS externalization could be a mechanism for removal of stress exposed young erythrocytes from circulation. This proposition was examined by studying the survival of control and stress exposed DIB labeled erythrocytes in blood. For this purpose, DIB labeled erythrocytes were further labeled with a green fluorescence dye CFSE and subjected to stress (8 h incubation in buffered saline containing 1 mM $CaCl_2$) before intravenous infusion in mice. At different time intervals, blood samples were taken and stained for biotin using SAv-APC and for PS by using annexin V-PE. Total surviving infused erythrocytes were gated flow cytometrically by using green fluorescence of CFSE. Within the whole surviving erythrocyte population, erythrocytes of different age groups as well as the proportions of PS$^+$ erythrocytes within different age groups could be assessed. Results in Fig. 5A show that stressed young erythrocytes were rapidly removed from circulation and only 40% of the infused young stressed erythrocytes survived in blood at 30 min post infusion. As the young stressed erythrocytes were selectively removed from the circulation, relative proportion of intermediate and old stressed erythrocytes within the recovered infused erythrocytes is expected to increase. This was confirmed since the proportions of intermediate and old stressed erythrocytes within the total

Table 1
Percentages of PS$^+$ erythrocytes in different age group of erythrocytes in control and macrophage depleted mice

Mice	Age group of erythrocytes			
	Unfractionated	<15 days	15–40 days	>40 days
Control	0.43 ± 0.15	0.44 ± 0.19	0.37 ± 0.20	0.46 ± 0.09
Macrophage depleted	0.37 ± 0.08	0.29 ± 0.11	0.36 ± 0.09	0.41 ± 0.15

Mouse erythrocytes were labeled with biotin by DIB technique (three injections of 1 mg BXN on day 1, 2 and 3, one injection of 0.6 mg BXN on day 28) and the mice were depleted of macrophages for 7 days by three i.v. administrations of clodronate loaded liposomes after the last injection of BXN as per the schedule described in Section 2. Blood samples were taken 1 day after the last injection of clodronate loaded liposomes. Percentages of PS$^+$ cells in different age groups of erythrocytes were determined by flow cytometry as described in Fig. 1. Each value represents mean ± SD from seven mice. PS expression on erythrocytes from control and macrophage depleted mice was not statistically significant in any group.

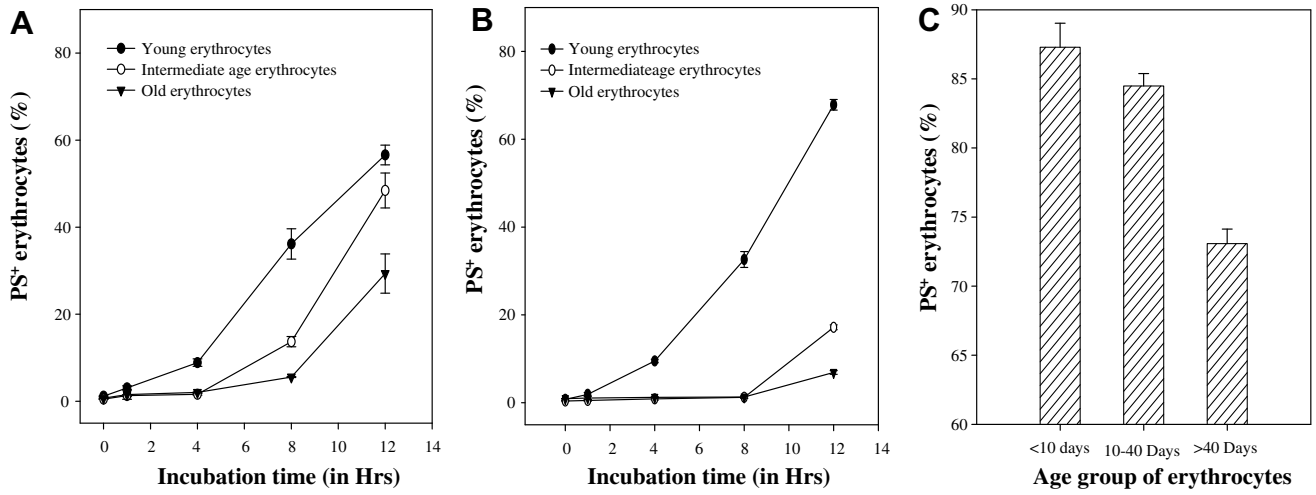

Fig. 3. Effect of various stresses on PS externalization on different age groups of erythrocytes. Mouse erythrocytes were biotin labeled by the DIB technique as in Fig. 1 except that the second biotinylation step was carried out 30 days after the first biotinylation step and blood was sampled 10 days after the second biotinylation step. This schedule enabled identification of erythrocytes belonging to young (<10 days), intermediate (10–40 days) and old (>40 days) age groups. Labeled 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 (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 (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 (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 (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.

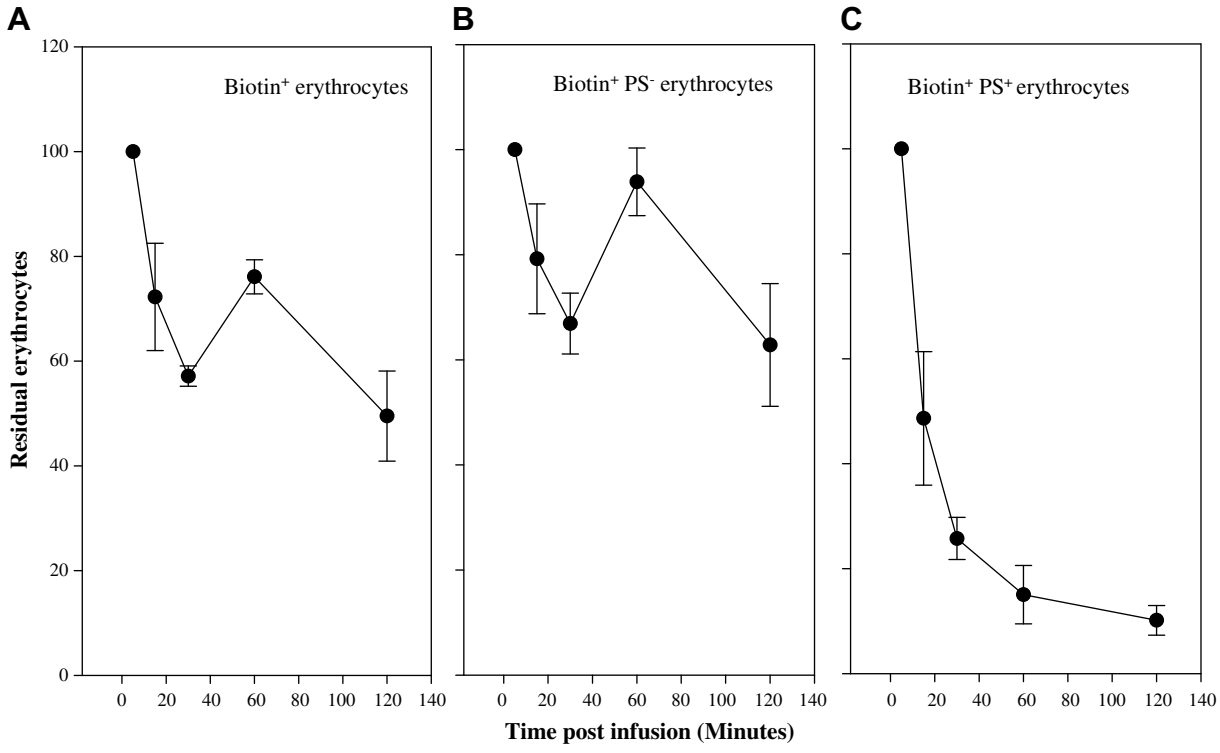

Fig. 4. Clearance of PS^+ erythrocytes from the blood circulation of mice. Mouse erythrocytes were biotin labeled *in vitro* and treated with calcium and calcium ionophore as described in Section 2, to induce PS externalization. These erythrocytes were infused in mice through tail vein and blood samples were collected after 5, 15, 30, 60 and 120 min. Infused $biotin^+$ erythrocytes in the blood were identified by staining with SAv PE. Within the $biotin^+$ erythrocytes, residual PS^+ and PS^- erythrocytes were examined by staining with annexin V FITC. Survival of $biotin^+$ (A), $biotin^+$ PS^- (B) and $biotin^+$ PS^+ (C) erythrocytes has been shown. Each point in the graph shows mean ± SD from four experiments.

infused population increased (120% and 140%, respectively, at 30 min time point). Percent PS^+ cells in surviving erythrocytes of different age groups at different time points after infusion are shown in Fig. 5B. These results clearly show that PS^+ young stressed erythrocytes were rapidly cleared from the blood.

4. Discussion

Phosphatidylserine (PS) externalization is an early event associated with apoptosis. Earlier studies in humans (Connor et al., 1994; Bratosin et al., 1998), rabbits (Boas et al., 1998) and mice (Manodo-

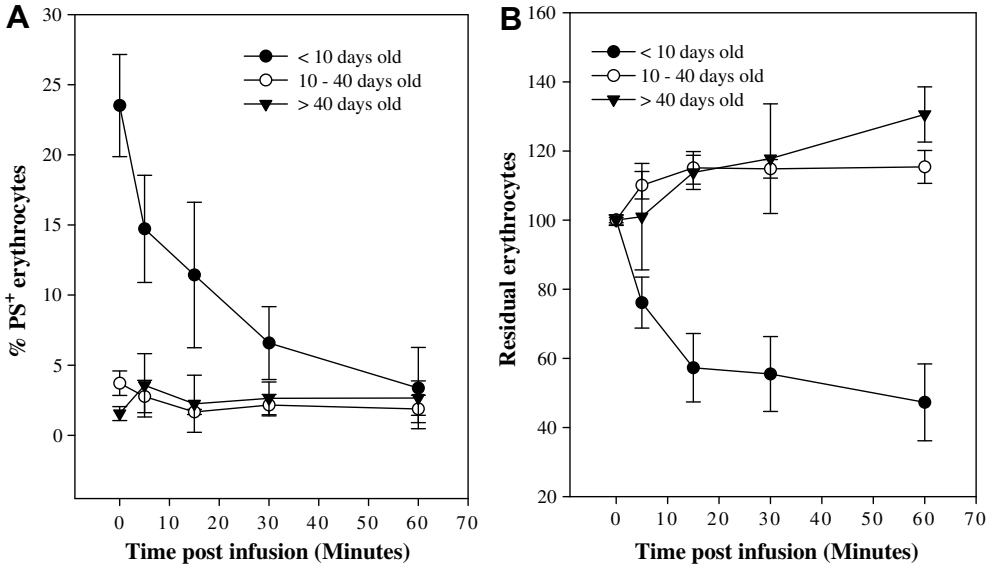

Fig. 5. Clearance of different age group of PS^+ erythrocytes from the circulation. Mouse erythrocytes were labeled using DIB technique as described in legend to Fig. 3. These erythrocytes were collected and stained with a green fluorescent dye CFSE and incubated for 8 h in the HEPES buffer saline with 1 mM $CaCl_2$. After *in vitro* treatment these erythrocytes were infused in a mouse and blood samples were collected at different time points. Erythrocytes were stained with SAv-APC and annexin V-PE and analyzed flow cytometrically. Residual erythrocytes ($CFSE^+$ population) were gated and proportion of different age group of erythrocytes and the PS^+ erythrocytes in each group was determined. (A) The change in the proportion of different age group of erythrocytes with reference to the initial time point. (B) The percentage of PS positive erythrocyte in different age group of erythrocyte population at different time point. Each point in the graph represents the mean ± SD of values obtained in three separate experiments. As compared to first time point, the decline in the numbers of infused erythrocytes (A) or PS^+ erythrocytes (B) in circulation was statistically significant (range $p < 0.05$–0.001) for the young (age <10 days).

ri and Kuypers, 2002) suggested a positive correlation in the PS exposure and age of erythrocytes suggesting that aged erythrocytes could be eliminated from the blood through a process resembling apoptosis. More recently however, Lutz (2004) and Arese et al. (2005) expressed reservations regarding the role of PS externalization in the normal clearance of erythrocytes from the circulation. Discrepancies in these results may be attributed to the fact that no good methods have been available for an unequivocal separation or identification of circulating erythrocytes of different ages. Techniques that have been used for separation of young and old erythrocytes are generally based upon changes that occur in physico-chemical properties of erythrocytes during the process of aging (Clark, 1988). Such properties however invariably have a substantial overlap between erythrocytes of different age groups. As a result, separation based upon such properties can at best yield erythrocytes enriched in young or old erythrocytes and an unequivocal separation of erythrocytes of different age groups is not possible. A system of serial hyper-transfusions of blood in the rat system to derive erythrocyte populations with increasing mean ages (Ganzoni et al., 1973), also did not permit analysis of erythrocyte cohorts of defined age groups.

Biotinylated erythrocytes were first used by Suzuki and Dale (1987) for studying the *in vivo* survival of these cells. Later, Hoffmann-Fezer et al. (1993) described a technique involving *in vivo* biotinylation of erythrocytes coupled with flow cytometry that could be used to objectively gate on young or old erythrocytes in blood and study their membrane phenotype. Using this technique an enhanced PS externalization on old erythrocytes was demonstrated in rabbits and mice (Boas et al., 1998; Manodori and Kuypers, 2002). Both these studies however utilized FITC (green fluorescence) labeled annexin V to stain PS^+ erythrocytes. Our recent results have shown that old populations of erythrocytes have a significantly greater green autofluorescence that can introduce artifacts in the interpretation of flow cytometric data if FITC labeled probes are utilized to compare expression of markers on young and old erythrocytes (Khandelwal and Saxena, 2007). This artifact is especially serious for studying changes in PS externalization because PS^+ erythrocyte populations are relatively very small (less than 3% in above mentioned studies). In the present study, we have used the new double *in vivo* biotinylation (DIB) technique to assess PS externalization on erythrocytes as a function of age, and found no evidence for an age dependent increase in proportion of PS^+ erythrocyte. APC (red fluorescence) in stead of FITC labeled annexinV was used in our studies to avoid the artifact that results from high green autofluorescence in old erythrocytes. Highest proportion of PS^+ erythrocytes (3–4%) was seen in youngest erythrocytes freshly released in blood. Percentage of PS^+ erythrocytes rapidly fell and remained below 1% throughout the remaining life span of erythrocytes. Relatively high proportions of PS^+ erythrocytes in very young age group could be due to residual PS expression after enucleation of hematopoietic precursors to reticulocytes, though we have no experimental evidence to support this proposition.

Macrophages in the reticulo-endothelial system are believed to be responsible for the clearance of aging erythrocytes from blood (Bennett and Kay, 1981; Clark, 1988). We have previously shown that *in vivo* depletion of macrophages by administration of clodronate loaded liposomes results in accumulation of old erythrocytes in circulation (Khandelwal et al., 2007). Low PS expression in oldest erythrocyte population could however not be boosted even by *in vivo* depletion of macrophages, indicating that the lack of accumulation of PS^+ erythrocytes was independent of macrophage clearance mechanism. Since PS externalization is not associated with normal aging of erythrocytes, it seems unlikely that it plays a significant role in removal of aged erythrocytes from the blood stream.

Our earlier studies have shown that the clearance of blood erythrocytes is not restricted to old erythrocytes and steady clearance of erythrocytes from blood circulation starts as early as ten days after their entry into the blood stream (Khandelwal and Saxena, 2006). Since the cell repair machinery is absent in erythrocytes, oxidative damage may accumulate in erythrocytes

irrespective of their age. Many stress conditions have been shown to induce PS externalization in the erythrocytes (Allan and Thomas, 1981; Bucki et al., 1998; Klarl et al., 2006). Our results show that the exposure of erythrocytes to stress resulted in PS externalization that is significantly more in young erythrocyte populations than in the old. The reason(s) for greater sensitivity of young erythrocytes to stress mediated PS externalization is not clear at present. Since erythrocytes lack nuclear machinery, they are unable to synthesize proteins needed for repairing any damage due to insults and stress. It is possible that the cellular machinery that brings about PS externalization in response to stress is gradually lost from erythrocytes as they get old. This proposition, however, needs to be further examined. Our results further demonstrate that after intravenous infusion, PS^+ as opposed to PS^- erythrocytes are rapidly cleared from blood circulation. These results suggest that PS externalization may indeed earmark erythrocytes for clearance. These results coupled with our observation that stress exposed young erythrocytes are significantly more prone to externalize PS than the similarly treated old erythrocytes, suggest that PS externalization could be an important mechanism for the clearance of erythrocytes damaged by stress. This hypothesis is supported by our results indicating that stress exposed young erythrocytes were cleared at a much faster rate than the old erythrocytes. Furthermore, within stress exposed young erythrocytes, PS^+ young erythrocytes were selectively removed from the circulation.

Taken together our results indicate that PS externalization on erythrocytes does not change significantly with the age of erythrocytes and therefore is unlikely to play a significant role in clearance of old erythrocytes from blood. PS externalization however occurs efficiently and predominantly in younger populations of erythrocytes exposed to stress and these PS^+ populations are rapidly cleared from blood circulation. PS externalization may therefore play an important role in the clearance of stress damaged young erythrocytes.

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