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ORIGINAL PAPER

Prolonged storage of red blood cells affects aminophospholipid translocase activity

A. J. Verhoeven, P. M. Hilarius, D. W. C. Dekkers, J. W. M. Lagerberg & D. de Korte

Sanquin Blood Supply Foundation, Division Research, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

Vox Sanguinis

Background and Objectives Loss of phospholipid asymmetry in the membrane of red blood cells (RBC) results in exposure of phosphatidylserine (PS) and to subsequent removal from the circulation. In this study, we investigated the effect of long-term storage of RBCs on two activities affecting phospholipid asymmetry: the ATP-dependent aminophospholipid translocase (or flippase, transporting PS from the outer to the inner leaflet) and phospholipid scrambling (which will move PS from the inner to the outer leaflet).

Materials and Methods Standard leukodepleted RBC concentrates were stored in saline-adenine-glucose-mannitol (SAGM) at 4 °C for up to 7 weeks. PS exposure was determined by measurement of AnnexinV-FITC binding to the cells, flippase activity by measurement of the inward translocation of NBD-labelled PS. Scrambling activity was determined by following the inward translocation of fluorescent NBD-phosphatidylcholine. In parallel, intracellular ATP levels were determined.

Results PS exposure amounted to only 1·5 ± 0·3% positive cells (n = 8) after 5 weeks of storage, which slightly increased to 3·5 ± 0·7% (n = 8) after 7 weeks of storage. Flippase activity started to decrease after 21 days of storage and reached 81 ± 5% of the control value after 5 weeks of storage (n = 6) and 59 ± 6% (n = 6) after 7 weeks. Also in RBC obtained by apheresis, flippase activity decreased upon storage. Scrambling activity remained virtually absent during storage, explaining the low PS exposure despite the decrease in flippase activity. Rejuvenation of RBC after 7 weeks to increase ATP levels only partially restored flippase activity, but in combination with a correction of the intracellular pH to that of fresh cells, almost complete restoration was achieved. The decrease in flippase activity after prolonged storage did make the RBCs more prone to PS exposure after activation of phospholipid scrambling.

Conclusion This study shows that, although PS exposure remains low, prolonged storage does compromise the RBC membrane by affecting flippase activity. When the metabolic changes induced by storage are corrected, flippase activity can be restored.

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Introduction

Freshly isolated erythrocytes exhibit an asymmetric distribution of phospholipids in their plasma membrane with the choline-containing lipids, phosphatidylcholine (PC) and sphingomyelin, predominantly in the outer leaflet, while the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS) reside mainly in the inner leaflet [1]. When PS or PE move to the outer leaflet, an ATP-dependent aminophospholipid translocase (or flippase) mediates inward translocation of these phospholipids [2] and thus prevents loss of phospholipid asymmetry. Another transporter (multidrug resistance protein 1, MRP1) has been suggested to play a role in outward movement of phospholipids [3], but in freshly isolated erythrocytes this activity is much lower than

Correspondence: A. J. Verhoeven, Department of Blood Cell Research, Sanquin Research, Plesmanlaan 125, 1066 CX Amsterdam, the Netherlands. Tel: 31-20-5123317; Fax: 31-20-5123310; E-mail: a.verhoeven@sanquin.nl

that of inward translocation. Hence, cycling of phospholipids [4] occurs at a very low rate. Only under some experimental conditions (e.g. elevation of intracellular Ca^{2+} or exposure to singlet oxygen) scrambling of phospholipids greatly increases and PS exposure becomes significant [5,6].

Red blood cells (RBC) destined for transfusion may, after isolation from donor blood, be stored for 5–7 weeks, depending on the prevailing regulations. Since the maintenance of phospholipid asymmetry is essential for cell survival and prevention of procoagulant activity, asymmetry should be maintained in red cell concentrates throughout storage. Two factors known to affect flippase activity – i.e. cellular ATP and intracellular pH [7] – do change during storage, but it is not well established whether the extent of changes in these parameters are relevant for stored RBC concentrates. In a pivotal study, Geldwerth *et al.* [8] used spin-labelled phospholipids to study phospholipid transport activities during long-term storage and showed that flippase decreases upon storage in citrate-phosphate-dextrose (CPD). The consequence of this decrease for exposure of PS could only be indirectly measured by sensitivity to hydrolysis by extracellular phospholipase A2, and appeared not to change during storage. Other investigators have used fluorescent probes to measure phospholipid movement [9,10] and PS exposure [5,6] in single cells by flow cytometry. With these probes, we have now re-investigated the effect of red cell storage on parameters determining phospholipid asymmetry. In our study, standard conditions for preparation and storage of red cell concentrates were used. Our results indicate that although PS exposure remains low during storage, the activity of the aminophospholipid translocase does decrease, in parallel with the decline in cellular ATP and intracellular pH. When the changes in ATP and pH in stored RBC are corrected by experimental manipulation, flippase activity can be restored.

Materials and methods

Materials

AnnexinV-fluorescein isothiocyanate (FITC) was from VPS-Diagnostics (Hoeven, the Netherlands). 1-Palmitoyl-2-[6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino] caproyl]-sn-glycero-3-phosphoserine (NBD-PS) and 1-palmitoyl-2-[6-[(7-nitro-2–1, 3-benzoxadiazol-4-yl)amino] caproyl]-sn-glycero-3-phosphocholine (NBD-PC) were from Avanti Polar Lipids (Alabaster, AL). Rejuvesol solution was obtained from enCyte Systems, Inc. (Braintree, MA). 1,9-Dimethyl-methylene blue (DMMB) was obtained from Aldrich (Steinheim, Germany).

Isolation of erythrocytes

Leucocyte-reduced erythrocytes were prepared by centrifugation of 500 ml whole blood (collected in CPD and stored for 12–18 h at 20–22 °C [11]) for 8 min at 2800 *g*. After removal of the buffy coat, 110 ml of the standard storage medium saline-adenine-glucose-mannitol (SAGM) [11] was added and in-line filtration of the erythrocyte suspension was carried out to remove residual leucocytes. For these procedures, blood collection systems with integrated leukoreduction filters were used (T3941, Fresenius Hemocare, Emmer Compascuum, the Netherlands). In some experiments, leucocyte-reduced erythrocytes were prepared by apheresis on the MCS + LN9000 system (Haemonetics Corp., Braintree, MA) according to the manufacturer's instructions with the LN942 disposable and the Single-Donor Red Cell Program. CPD-50 was used as anticoagulant and SAGM as additive solution. After preparation, the apheresis units were leukodepleted as described above.

The resulting erythrocyte suspensions had a volume of 275–320 ml, a haematocrit (Ht) of about 60% and contained $< 1 \times 10^6$ leucocytes per unit (as determined with a Nageotte haemocytometer), whereas platelet counts were below detection limit (determined with an AcT10, Beckman Coulter, Mijdrecht, the Netherlands). After preparation, the red cell concentrates were stored in 600 ml PVC storage bags (Fresenius Hemocare) at 4 °C in a standard blood bank refrigerator.

AnnexinV labelling of erythrocytes

To quantify the amount of erythrocytes exposing PS on their cell surface, cells were stained with FITC-labelled AnnexinV, essentially according to Kuypers *et al.* [5]. Samples of red cells, taken aseptically from RBC storage bags, were washed twice with an incubation medium consisting of 134 mM NaCl, 10 mM glucose, 10 mM Tris-HCl, and 40 mM sucrose (SGT/sucrose), and supplemented with 20 mM Hepes and 0·5% human serum albumin (HSA) (pH 7·4). After washing, the cells were resuspended at 0·3% Ht in the same medium supplemented with 2·5 mM $CaCl_2$. Labeling with AnnexinV was performed by adding AnnexinV-FITC (final concentration 1 μg/ml) to 250 μl cell suspension. After incubation at room temperature (RT) in the dark for 30 min, cells were washed once and analysed on a Becton Dickinson FACScan flow cytometer. Data analysis was performed using the WinMDI 2·8 software program (Scripps Research Institute, La Jolla, CA). The percentage of AnnexinV-positive cells was determined by comparison with a negative control incubated with goat antimouse IgG-FITC (1 μg/ml).

Measurement of flippase and scrambling activity

Flippase was measured in a flow cytometer by determining the inward translocation of NBD-PS [10] and scrambling activity was measured by determining the inward translocation of NBD-PC [6]. For both analyses, samples of red cells taken aseptically from RBC storage bags were washed twice in SGT/sucrose (pH 7·4) and resuspended to 5% Ht (equivalent

with $5 \cdot 10^8$ cells/ml) and incubated for 5 min at 37 °C in a volume of 0·5 ml. The extracellular pH, as measured at 37 °C in a blood gas analyser (Rapidlab 860, Bayer, Mijdrecht, NL) was 6·86 under these standard incubation conditions. The assay was started by the addition of 5 μl NBD-phospholipid (200 μm, final concentration 2 μm). These stock solutions of NBD-PC or NBD-PS were freshly prepared by drying a stock solution in $CHCl_3$ and subsequent resuspension in SGT/sucrose. At different time intervals, 20-μl aliquots were removed from the cell incubations and diluted in 1 ml ice-cold SGT/sucrose with 1% BSA to extract label present in the outer leaflet of the membrane [8,10]. To measure total fluorescence, samples were mixed with SGT/sucrose in the absence of BSA. After at least 5-min extraction time on ice, samples were analysed on a Becton Dickinson FACSAN flow cytometer, measuring forward scatter, side scatter and green fluorescence. The data were analysed using WinMDI 2·8. The fraction of probe translocated was determined by dividing the mean arithmetic fluorescence of the sample population after BSA extraction (internalized probe) by the mean fluorescence of the same population in the absence of BSA (total probe bound). Analysis of the cell supernatant indicated that virtually all the label added was bound by the cells. The integrity of the NBD-probes was verified with thin-layer chromatography. No degradation of the probes was observed during the course of the experiments.

Measurement of ATP levels in erythrocytes

Samples of red cell concentrates suspended in SAGM (0·6 ml) were diluted with 0·9 ml of phosphate-buffered saline (PBS, pH 7·4) and then acidified with 60 μl perchloric acid (70%, w/v). After 10 min on ice, extracts were centrifuged in the cold for 5 min at 12 000 ***g*** and 1 ml of protein-free supernatant was neutralized with 56 μl 5 N K_2CO_3. When cells had been pretreated to alter ATP levels, cells adjusted to an Ht of 25% were extracted without dilution with PBS. Samples were frozen at −30 °C. After thawing, samples were homogenized and centrifuged to remove $KClO_4$ precipitate and ATP was measured in the clarified supernatant by adding to a mixture of 10 mM glucose, glucose-6-phosphate dehydrogenase (G6PD, 0·7 U/ml) and 0·5 mM $NADP^+$. After reading the absorption at 340 nm in a 96-well microtitre plate, conversion of ATP was started by addition of hexokinase (5 U/ml). ATP conversion was complete within 10 min. Changes in absorbance due to NADPH formation were calibrated by addition of known standards of ATP (0–200 μm).

Haemolysis

Free haemoglobin in the different erythrocyte suspensions was determined by absorbance measurement of cell supernatants at 415 nm, with correction for plasma absorption. Cell supernatants were obtained by centrifugation of the erythrocyte suspensions for 2 min at 12 000 ***g***. Haemolysis was expressed as percentage of total haemoglobin present in erythrocyte lysates after correction for Ht.

Measurement of intracellular pH

Intracellular pH was measured essentially according to Meryman *et al.* [12]. In short, 1-ml samples of RBC were centrifuged for 2 min at 12 000 ***g*** and the dry pellets were quickly frozen in liquid nitrogen. After thawing, 200–500 μl of aqua dest was added and the pH of the resulting lysate was measured at 37 °C in a blood gas analyser (Rapidlab 860, Bayer, Mijdrecht, NL).

ATP depletion, rejuvenation and pH correction of erythrocytes

In some experiments, erythrocytes from freshly isolated concentrates were washed in 134 mM NaCl, 10 mM Tris-HCl, and 40 mM sucrose (ST/sucrose, pH 7·4) and subsequently incubated (Ht 10%) for 4 h at 37 °C in the presence of 10 mM glucose to maintain intracellular ATP levels or in the presence of 5 mM 2-deoxyglucose (DOG) to partially deplete intracellular ATP. Subsequently, the cells were diluted in the same medium to an Ht of 3% and flippase activity was measured as described, except that the amount of NBD-PS was lowered to 1·2 μM.

In other experiments, erythrocyte suspensions from stored concentrates were treated with Rejuvesol [13], a solution containing 100 mM pyruvate, 100 mM inosine, 100 mM NaPi and 5 mM adenine (pH 7·0) in order to increase intracellular ATP levels. For this, 5 ml red cell concentrate was mixed with 1 ml Rejuvesol solution and incubated for 30 min at 37 °C. Control samples were incubated at 37 °C without addition of Rejuvesol. Subsequently, the cells were washed and resuspended in SGT/sucrose to an Ht of 25%. After sampling for ATP determination, the cells were further diluted in SGT/sucrose to an Ht of 5% and flippase and scrambling activity was measured as described above. Samples of stored RBC, either untreated or rejuvenated, were also incubated for 15 min in SGT/sucrose with a higher pH (pH 7·7 instead of 7·4, resulting in a pH of 7·12 instead of 6·86 as measured at 37 °C in the blood gas analyser) prior to measurement of flippase activity.

Photodynamic treatment

In some experiments scrambling activity was induced by exposure of erythrocyte suspensions to singlet oxygen by photodynamic treatment with DMMB as described previously [6]. In short, cell suspensions were washed, resuspended in SGT/sucrose to an Ht of 3% and incubated for 10 min at room temperature with 20 μm DMMB as photosensitizer. Subsequently, aliquots of cells (300 μl) were placed in polystyrene cell culture plates with 48 flat-bottom wells (Corning Inc.,

Table 1 PS exposure and NBD-PC translocation activity in fresh and stored erythrocytes

Erythrocytes	Haemolysis (%)	PS exposure (% positive cells)	NBD-PC translocation (% uptake in 60 min)
Day 1	0·02 ± 0·00 (8)	0·6 ± 0·1 (8)	0·4 ± 0·3 (4)
Day 35	0·15 ± 0·03 (8)	1·5 ± 0·3 (8)[a]	1·2 ± 0·3 (4)[b]
Day 49	0·26 ± 0·05 (8)[d]	3·5 ± 0·7 (8)[d]	1·5 ± 0·1 (4)[c]

Samples of red cell concentrates stored at 4 °C were taken at the time points indicated and analysed for haemolysis, PS exposure and NBD-PC translocation (as measure of scrambling activity) as described in the Materials and methods. Results given are the mean ± SEM of the number of observations given in parentheses. [a]$P < 0.05$, as compared to day 1 (Student's *t*-test for paired observations); [b]$P < 0.02$, as compared to day 1 (Student's *t*-test for paired observations); [c]$P < 0.01$, as compared to day 1 (Student's *t*-test for paired observations); [d]$P < 0.005$, as compared to day 1 (Student's *t*-test for paired observations).

Corning, NY) and illuminated with red light (λ > 560 nm) on a rocking platform with a fluency of 15 MW/cm^2 for 8 min. The light dose amounted to 75 kJ/m^2. After phototreatment, the cells were analysed for scrambling activity and AnnexinV binding as described above.

Results

As reported earlier, freshly isolated erythrocytes contain very little, if any, PS-positive cells (< 1%, see Table 1). After storage in SAGM at 4 °C for 5 weeks, which is the maximum period of storage allowed in the Netherlands, the percentage of PS-positive cells had increased slightly to 1·5%. After another 2 weeks of storage, the percentage of PS-positive cells further increased, but still most units remained below 5%. Haemolysis increased only very little during the entire period of storage (Table 1).

In a previous study [6] we showed that scrambling activity, as measured by the inward translocation of NBD-labelled phosphatidylcholine, is undetectable in freshly isolated erythrocytes and may only be detected after exposure of the cells to singlet oxygen, apart from the known activation by elevation of cytosolic free Ca^{2+} [14]. In this study, we investigated whether scrambling activity increases during storage, but our data strongly indicate that scrambling activity remains very low, even after 7 weeks of storage (Table 1).

One of the other components contributing to phospholipid asymmetry is the aminophospholipid translocase or flippase. In freshly isolated erythrocytes, uptake of NBD-labelled PS was almost linear in the first 30 min of incubation at 37 °C (Fig. 1). Translocation of NBD-PS was completely absent in the presence of N-ethylmaleimide (NEM), a known inhibitor of flippase activity [15].

During storage, flippase activity was measured by determining the uptake of NBD-PS after 15 and 30 min of incubation at 37 °C (i.e. in the initial phase of NBD-PS translocation). The results depicted in Fig. 2 show that flippase activity (as judged from 30-min translocation of NBD-PS) significantly declined

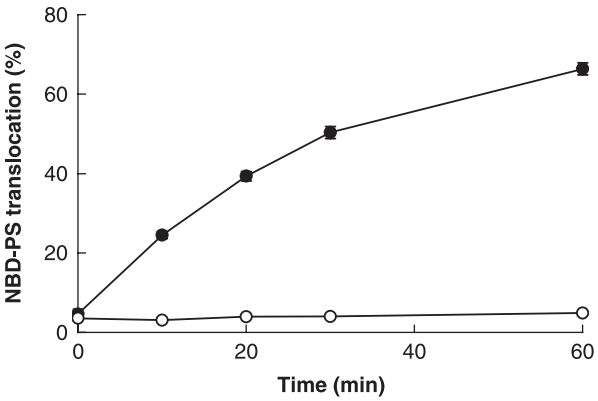

Fig. 1 Time course of NBD-PS translocation as measure of flippase activity. Erythrocytes suspended in SGT/sucrose (Ht 5%) were incubated at 37 °C in the absence (closed circles) or presence of 10 mm NEM (open circles). After 5 min ($t = 0$ in the figure), 2 μM NBD-PS was added and samples were taken at different time points. Translocation of NBD-PS was measured by flow cytometry as described in the Materials and methods. Results given are the mean ± SD of three independent experiments. Error bars of several data points are smaller than 0·5%.

after 28 days of storage. After 5 weeks of storage, activity had decreased to 81 ± 5% of the starting value and after 7 weeks to 59 ± 6% (mean ± SEM, $n = 6$). A similar decrease was observed when the data of the 15-min translocation period were compared (data not shown). Since flippase activity is ATP-dependent, cellular ATP levels were measured in parallel. Figure 2 shows that ATP levels declined with similar kinetics as the activity of flippase. In fact, when the data on flippase activity depicted in Fig. 2 were replotted against the ATP levels measured, a very good correlation between the two parameters was obtained (Fig. 3).

The data shown in Fig. 2 on the effect of storage on flippase activity were obtained with standard red cell concentrates routinely prepared in the Netherlands from buffy-coat depleted whole blood and stored in SAGM. One may argue that these results were influenced by the depletion of erythrocytes

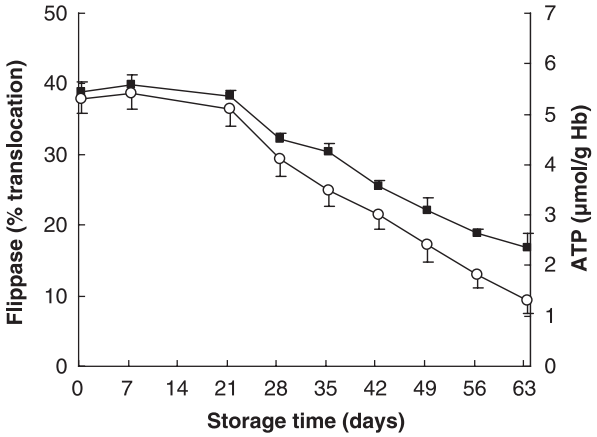

Fig. 2 Flippase activity and ATP levels during storage of red cell concentrates. Samples were taken at different time points of red blood cell storage and analysed for flippase activity measured in a 30-min incubatiuon at 37 °C (closed squares) and ATP levels (open circles), as described in the Materials and methods. Results given are the mean ± SEM of four to six red cell concentrates stored in SAGM.

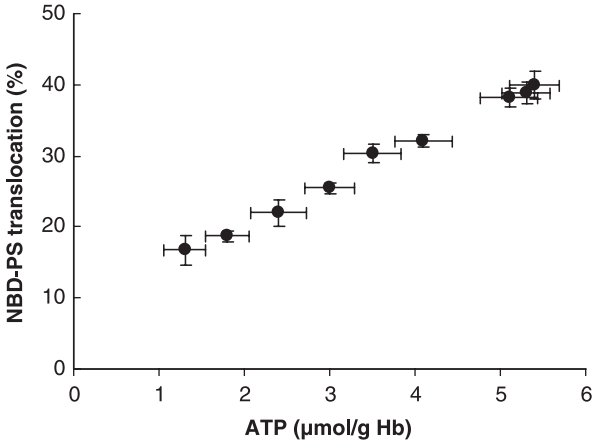

Fig. 3 Correlation of flippase activity and ATP levels during storage of red cell concentrates. Data on flippase activities during red cell storage as depicted in Fig. 2 were replotted against ATP levels measured in the same samples. Results given are the mean ± SEM of four to six red cell concentrates stored in SAGM.

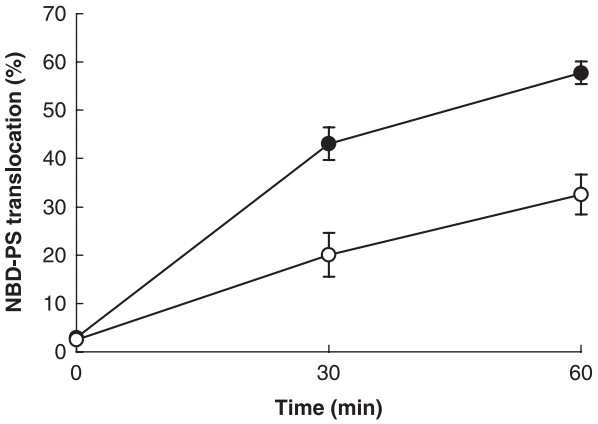

Fig. 4 Effect of ATP depletion in fresh erythrocytes. Freshly isolated erythrocytes were incubated for 4 h at 37 °C in the presence of glucose (control, closed symbols) or in the presence of 2-deoxyglucose (DOG, open symbols) as described in the Materials and methods. Intracellular ATP in the presence of DOG decreased to 45·6 ± 7·5% (mean ± SEM of four observations) of control levels in the presence of glucose. Subsequently, flippase activity was measured by following NBD-PS translocation in time. Results given are the mean ± SEM of four independent experiments.

Table 2 Flippase activity and ATP levels during storage of different red cell concentrates

Erythrocytes	NBD-PS translocation (% uptake in 30 min)	ATP (µmol/g Hb)
Day 1		
Apheresis RCC	51·6 ± 0·9[a]	3·8 ± 0·2[b]
BC-depleted RCC	38·8 ± 1·5	5·3 ± 0·3
Day 35		
Apheresis RCC	27·2 ± 0·7[c]	2·5 ± 0·1[a]
BC-depleted RCC	30·3 ± 1·2	3·5 ± 0·3

Red cell concentrates prepared by apheresis or by standard buffy coat (BC) depletion of whole blood were analysed at day 1 and day 35 of storage at 4 °C for NBD-PS translocation (as measure of flippase activity) and ATP levels, as described in the Materials and methods. Results given are the mean ± SEM of six preparations. The data for the standard BC-depleted units were derived from the experiments depicted in Fig. 2.
[a]$P < 0{\cdot}0001$, as compared to BC-depleted RCC (Student's *t*-test for unpaired observations); [b]$P < 0{\cdot}0005$, as compared to BC-depleted RCC (Student's *t*-test for unpaired observations); [c]$P < 0{\cdot}05$, as compared to BC-depleted RCC (Student's *t*-test for unpaired observations).

of relative low density and hence of relative young age [9]. We therefore investigated flippase activity in red cell concentrates obtained by apheresis (Table 2). Although the initial flippase activity was about 30% higher in the apheresis units than in the buffy-coat depleted units, flippase activity also decreased during storage with a concomitant decrease in ATP levels.

Although the data in Table 2 indicate that in apheresis units the relation between flippase activity and ATP was different from that in buffy-coat depleted units, the storage-induced decrease in ATP may have been important for the decline in flippase activity we observed. Two approaches were taken to substantiate this hypothesis. First, in freshly isolated erythrocytes ATP levels were down-regulated to a similar extent as induced by prolonged storage. A 4-h incubation at 37 °C in the presence of deoxyglucose proved to be sufficient to reach this goal. As in stored erythrocytes, the decline in ATP to about 50% of the control value correlated with a similar decrease in the rate of NBD-PS uptake (Fig. 4).

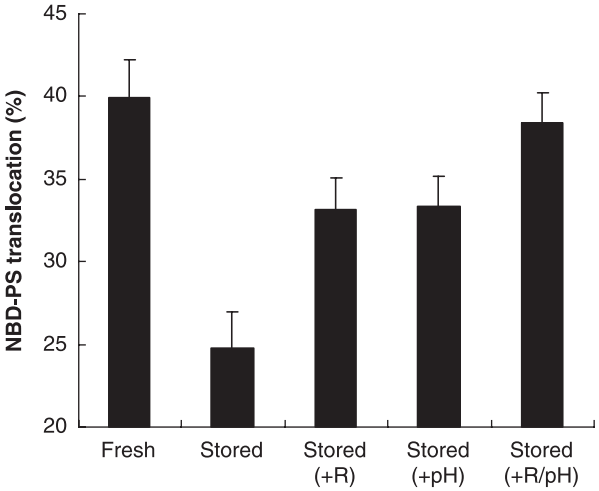

Fig. 5 Effect of rejuvenation and pH manipulation on flippase activity in stored erythrocytes. Samples of red cell concentrates, freshly isolated (fresh) or stored for 7 weeks at 4 °C (stored), were treated with Rejuvesol (+R) or incubated at higher pH (+pH) as described in the Materials and methods. NBD-PS translocation was measured for 30 min at 37 °C to determine flippase activity. The ATP levels (in µmol/g Hb) amounted to 4·70 ± 0·21 in case of fresh cells, 1·64 ± 0·19 in stored cells, and 5·30 ± 0·61 in rejuvenated cells. Intracellular pH amounted to 6·83 ± 0·01 in case of fresh cells, 6·36 ± 0·02 in stored cells, and 6·82 ± 0·01 in stored cells incubated at higher pH. Rejuvenation increased intracellular pH by less than 0·1 unit. Results given are the mean ± SEM of 5 red cell preparations. Statistical significance of differences (as compared with fresh cells by Student's *t*-test): $P < 0.0005$ for stored cells, $P < 0.02$ for stored (+R)-cells and stored (+pH)-cells, and $P > 0.3$ for stored (+R/pH)-cells.

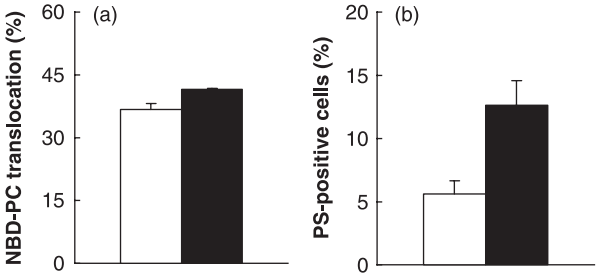

Fig. 6 Effect of photodynamic treatment on fresh and stored erythrocytes. Samples of red cell concentrates, freshly isolated (open bars) or stored for 7 weeks at 4 °C (closed bars) were treated with DMMB and red light as described in the Materials and methods. Subsequently, cells were analysed for scrambling activity (a) and PS exposure (b). Results given are the mean ± SEM of four independent observations.

Second, erythrocytes stored for 7 weeks were incubated with a rejuvenation solution [13] in order to increase cellular ATP levels. Although the rejuvanation procedure was successful in restoring ATP levels, flippase activity did not return completely to the value observed in fresh erythrocytes (Fig. 5, third bar). The latter result indicates that, apart from ATP levels, another factor is involved in the decline in flippase activity upon prolonged storage.

As a candidate for another factor affecting flippase activity, we considered changes in intracellular pH. Intracellular pH decreases during storage [16] and Libera *et al.* [7] have shown that flippase activity decreases with decreasing pH. Indeed, in the washed RBC suspensions tested for flippase activity, the intracellular pH was 6·83 ± 0·01 when freshly isolated RBC concentrates were used, and 6·36 ± 0·02 ($n = 5$, mean ± SEM) when RBC were used that had been stored for 7 weeks. When the stored RBC suspensions were incubated in a buffer with a higher pH to correct for this difference, flippase activity indeed was higher (Fig. 5, fourth bar). When rejuvenation and higher pH were combined, flippase activity increased almost completely to values observed in freshly isolated RBC (Fig. 5, fifth bar).

In the last set of experiments, we investigated the possible consequences of the decline in flippase activity upon prolonged storage. The data presented in Table 1 already indicate that under normal storage conditions this decline does not result in severe loss of phospholipid asymmetry, but the data depicted in Fig. 2 show that stored red cells may become compromised with respect to their ability to prevent PS exposure. To test this possibility, fresh and stored erythrocytes were exposed to photodynamic treatment, inducing singlet oxygen known to induce scrambling activity [6]. Although scrambling activity was induced to a similar degree in fresh cells and in cells stored for 7 weeks (Fig. 6a), the resulting exposure of PS was significantly higher in the stored cells (Fig. 6b). In red cells stored for 5 weeks, PS exposure was similarly induced as in fresh cells (data not shown). These results suggest that stored cells, at least after prolonged storage, are more limited in compensating outward movement of PS.

Discussion

The present study, carried out with fluorescent probes to assess characteristics of phospholipid transport in stored erythrocytes, underscores the previous observations of Geldwerth *et al.* [8] obtained with spin-labelled probes in CPD-stored units (i.e. a decline of flippase activity during prolonged storage without a significant loss of phospholipid asymmetry). However, our results were obtained in red cell concentrates stored in SAGM under standard blood bank conditions. The decline in flippase activity and the increase in scramblase activity (as measured by NBD-PC translocation) were less severe, probably due to the optimized conditions of storage at 4 °C as indicated by the low degree of haemolysis (Table 1). Flippase acitivity also decreased in units obtained by apheresis, although the initial activity was higher, possibly reflecting a higher contribution of relatively young erythrocytes [9].

In fact, the changes in flippase activity induced by prolonged storage of red cell concentrates are reminiscent of the difference in flippase activity between young and aged erythrocytes when these populations are separated from a fresh blood sample by density gradient centrifugation [9]. This suggests that during *in vitro* storage changes occurring *in vivo* may continue and amplify. Indeed, when erythrocytes from stored concentrates were subjected to Percoll gradient separation, more cells were recovered from the more dense fraction (results not shown). In our study, we observed that the storage-induced decrease in flippase activity could largely be overcome by correcting the metabolic changes that occur during storage. It remains to be determined whether this is also observed when erythrocytes aged in the circulation are subjected *in vitro* to the same metabolic corrections.

The decline of flippase activity, which is observed even under standard conditions of storage, is accompanied by changes in ATP levels and intracellular pH. Both the study of Geldwerth *et al.* [8] and the present one show that flippase activity falls when ATP levels decrease below the physiological level of 5 μmol/g Hb (equivalent to a cytosolic concentration of about 1·5 mM). However, the correlation shown in Fig. 3 suggests that ATP may not be the determining factor itself. The dependence does not show a classical Michaelis-Menten relationship and flippase activity still increases at ATP concentrations higher than 1 mM, which is significantly higher than the K_m value of 250 μm observed in partially purified flippase preparations [17]. This apparent discrepancy may be caused by binding of ATP in the cytosolic compartment or by a change in affinity upon solubilization of the enzyme. It should also be noted that in erythrocytes obtained by apheresis the relation between flippase activity and ATP seems to be different (Table 2). The changes in ATP levels may therefore reflect changes in another metabolite important for flippase activity or reflect the concurrent changes in intracellular pH.

At first sight, these results do not seem to warrant important changes in current practices of red blood cell storage. The exposure of phosphatidylserine, which would compromise the survival of transfused cells [18], remains very limited during 5 weeks of storage and stays below 5% even after 7 weeks of storage. It should be noted that in our measurements microvesicles were not included, which also might contribute to procoagulant activity in stored red cell concentrates. However, the extent of microvesiculation in our standard SAGM products was only very limited: on average 5% of the haemoglobin in cell-free supernatants appeared to be associated with microvesicles (data not shown).

The underlying mechanism for the good maintenance of phospholipid asymmetry is most likely the virtual absence of phospholipid scrambling throughout the storage period. Candidate genes responsible for phospholipid scrambling have been cloned [19,20], but a knockout mouse of the first member of this gene family showed unchanged scrambling in Ca^{2+}-activated red cells [21]. Thus, although the enzyme remains elusive, it is apparent that activating signals for scrambling are not present under standard storage conditions of red cell concentrates for 35 days. Nevertheless, our study also shows that under conditions of stress, prolonged storage does compromise the cells in their ability to maintain phospholipid asymmetry, and therefore measures to prevent this seem to make sense.

The current quality requirements for stored red cell concentrates are rather limited. In general, haemolysis must stay below 0·8% or 1%, depending on the prevailing regulations. Extent of haemolysis, however, is not a good indicator of the metabolic fitness of red cells as exemplified, for instance, by the fact that haemolysis does not increase by more than 0·3% during the 7-week storage in SAGM (Table 1). It seems preferable to require a minimum level of cellular ATP, because this parameter correlates not only with flippase activity but also, at least to a certain extent, with *in vivo* recovery [22]. From the data published by Heaton *et al.* [22] it can be derived that ATP levels should be higher than 2·7 μmol/g Hb to have a greater than 90% chance for a 24-h recovery of 75% or higher. The way cellular ATP influences this important parameter has not been clearly defined. It is tempting to speculate that a decrease in flippase activity, limiting the ability of the cells to counteract outward movement of PS, might contribute to the way ATP (or a related metabolite) influences red cell survival. The most attractive way to maintain ATP levels is to use storage media, in which the decrease in intracellular pH during storage is prevented [12,16]. Storage media promoting maintenance of intracellular pH would have an additional beneficial effect, because, as this and previous studies [7] have shown, pH itself is an important determinant for flippase activity.

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