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Alteration of the aminophospholipid translocase activity during in vivo and artificial aging of human erythrocytes

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Human erythrocytes were separated into three density groups representing different age groups. Phospholipid outside-inside translocation rates and equilibrium distribution were determined in each group with spin-labeled phosphatidylserine (PS*), phosphatidylethanolamine (PE*), and phosphatidylcholine (PC*), at 37°C and 4°C. At both temperatures, the initial velocity of aminolipid translocation was reduced in the more dense (older) cells. The equilibrium distribution was not significantly modified for PS*, but a larger fraction of PE* remained on the outer monolayer of the more dense cells. PC* transmembrane diffusion was identical in the three fractions. Cytosolic ATP, which is required for aminophospholipid translocation, was not responsible for the variability of the density separated cells since ATP enrichment did not cancel the differences between top and bottom fractions, although it equalized the ATP concentration of the various fractions. Variations in the level of intracellular Ca^{2+} could also be excluded. Thus, the enzyme aminophospholipid translocase seemed to be directly altered in aged cells, possibly due to oxidation caused by lipid peroxidation products. Experiments with malonyldialdehyde or H_2O_2 treated cells confirmed this interpretation and suggest that defects in endogenous lipid asymmetry observed in aged human erythrocytes may be due to altered activity of the translocase.

Introduction

It is now well established that phospholipid distribution across the erythrocyte membrane is asymmetrical: phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly present in the outer monolayer while almost all of the phosphatidylserine (PS) and most of the phosphatidylethanolamine (PE) are present on the inner side of the membrane bilayer [1,2]. Using in vitro manipulations of lipid asymmetry, several laboratories have demonstrated that erythrocyte adhesion to other cells, such as monocytes or endothelial cells, is increased when PS appears on the outer monolayer [3–6]. Thus, it was proposed by Tanaka and Schroit in 1983 [3] that PS on the outer monolayer of aged erythrocytes might serve as a signal for triggering their recognition by macrophages. The abnormally high level of PS on the outer leaflet of sickled cells could be the cause of their relatively short life-time in the blood stream [7]. The mechanism by which this cell–cell interaction proceeds may be a direct recognition of the PS head group as suggested by the binding to macrophages of PS containing liposomes [8]. Alternatively, PS on the outer monolayer of erythrocytes may interact with the protein carrying the 'senescent antigen' [9–11], revealing it to the macrophages. It remains to be understood why in normal erythrocytes the transmembrane lipid asymmetry would be lost after approximately 120 days, which corresponds to the life-time of red blood cells.

The major cause of phospholipid asymmetry appears to be the activity of a specific phospholipid pump, the aminophospholipid translocase [12,13] (for a review, see Ref. 2 and reference therein). It has also been suggested that cytoskeleton proteins would contribute to the lipid asymmetry because of specific phospholipid interactions [14,15]. A change in the transmembrane equilibrium distribution can be due to an accelerated lipid

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Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PS*, spin-labeled phosphatidylserine; PE*, spin-labeled phosphatidylethanolamine; PC*, spin-labeled phosphatidylcholine; RBC, red blood cell; MDA, malonyldialdehyde; BSA, bovine serum albumin; ESR, electron spin resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide.

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flip-flop and/or a degradation of the proteins involved in the establishment and maintenance of lipid asymmetry. The present study was undertaken to examine the activity of the aminophospholipid translocase in in vivo aged RBC using a spin-label technique previously developed in our laboratory [16]. In complementary experiments, we have made the comparison with the loss of translocase activity which can be induced by incubation with malonyldialdehyde (MDA) or H_2O_2. The influence of ATP and calcium was also examined.

Materials and Methods

Fresh human blood was obtained from healthy volunteers or from a local blood bank (Fondation Nationale de Transfusion Sanguine). Blood collected on EDTA was stored at 4° C and used within 5 days.

Separation of erythrocyte into different age groups. First method: This method introduced by Murphy [17], and modified by Shukla and Hanahan [18], consists essentially of an ultracentrifugation in cell free blood plasma, except that, to avoid contamination with reticulocytes, the upper 5% of each tube after final centrifugation was discarded [19]. The rest of each tube was divided into top (≈ 10%), bottom (≈ 10%) and middle fractions. The buffer used to resuspend the cells was: 145 mM NaCl, 5 mM KCl, 5 mM Na_2HPO_4, 20 mM Hepes, 1 mM $MgSO_4$, 0.1 mM EGTA, 10 mM glucose, pH 7.4 (buffer A). Second method: The separation of erythrocytes by Percoll density gradient was performed as described by Lutz and Fehr [20]. 500 ml of Percoll medium contained 378 ml of Percoll (Pharmacia), 19 ml 3 M NaCl, 0.5 g of glucose and 25 ml of 0.2 M, Na_2HPO_4/KH_2PO_4 (pH 7.4) 10 vol. of pelleted erythrocytes were layered on 32 vol. of Percoll medium and then centrifuged 22 min at 4° C, at 43000 × *g* with an angle rotor (Beckman 45TI). The cells formed bands in the tube and were removed in three fractions as described by Lutz and Fehr [20]. The upper 5% was discarded to avoid contamination by reticulocytes; next 5% constituted the 'top' fraction, the 'middle' and the 'lower' fractions corresponded to the middle band (10%) and the 3 lower bands (2–3%), respectively. Each fraction was washed three times with buffer A to remove Percoll.

Shape of fractionated cells. 2 h after incubation of labeled cells at 37° C, 20 µl of RBC were added to 300 µl of 1% glutaraldehyde in buffer A and allowed to stand 1 h on ice. Before observation under a light microscope (Zeiss) the cells were diluted 100-fold.

ATP enrichment. The cells were suspended to 10% hematocrit in buffer B (60 mM NaCl, 40 mM KCl, 50 mM Na_2HPO_4, 10 mM pyruvate, 10 mM adenosine, 10 mM inosine, 10 mM glucose, 1 mM $MgCl_2$ (pH 7.4) and incubated at 37° C for 4 h.

Increase of cytosolic Ca^{2+}. Erythrocytes were washed twice in buffer C: 70 mM KCl, 70 mM NaCl, 10 mM Hepes, 2 mM $MgSO_4$, 1 mM EGTA, 20 mM inosine, 10 mM glucose (pH 7.4). Then 0.5 vol. of pelleted cells was added to 4.5 vol. of buffer C (≈ 10% hematocrit) containing Ca^{2+} at the desired external concentration. A23187 (Sigma) dissolved in DMSO (5 mM stock solution) was added at a final concentration of 10 µM. A minimum incubation of 10 min at 37° C was allowed for equilibration. The intracellular Ca^{2+} concentration was calculated as described in Ref. 21.

MDA treatment of erythrocytes. MDA was freshly prepared as described in Ref. 22. Erythrocytes washed twice in buffer A were incubated at 37° C (≈ 5% hematocrit), for 1.5 h at the desired MDA concentration. After incubation the cells were washed four times in buffer A.

H_2O_2 treatment of erythrocytes. After two washes in buffer A, erythrocytes were incubated at 5% hematocrit, at 37° C for 30 min in buffer A in the presence of 1 mM NaN_3, a catalase inhibitor [23]. NaN_3 was present during all further steps. Control experiments showed that NaN_3 had no effect on translocation of spin-labeled phospholipids. H_2O_2 at various concentrations was added at 37° C and cells incubated 1.5 h (≈ 5% hematocrit). At the end of the incubation, erythrocytes were washed four times in buffer A.

Spin-labeling and ESR determination of the transmembrane distribution of spin-labeled lipids. Before labeling, each membrane fraction was incubated 5 min with 5 mM (final concentration) diisopropyl fluorophosphate (DFD), at 37° C, to minimize the hydrolysis of the spin-labeled phospholipids [24]. 1-Palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylcholine (PC*), -phosphatidylserine (PS)*) and -phosphatidylethanolamine (PE*) were synthesized as described in Refs. 12 and 16. The spin-labels were resuspended by vigourous vortexing in buffer A before incubation with the membranes. Labeling of the erythrocytes was carried out at an hematocrit of 33%, which guarantees an homogeneous incorporation of the exogenous lipids. The molar ratio of spin-label to endogenous phospholipid in the mixture was maintained below 1%, in order to avoid lytic effects due to the amphiphilic character of the short chain phospholipids. The ESR determination of the transmembrane distribution of spin-labeled lipids was performed by the back exchange technique as described by Morrot et al. [16]. ESR measurements were performed with a Varian E-109 spectrometer equipped with a temperature control device and connected to a Tektronix computer for accumulation and averaging of the spectra.

Miscellaneous. Phospholipid concentration was determined by the method of Rouser et al. [25]. ATP determination was performed by the luciferin-luciferase assay [26].

Results

Density separated RBC

Two classical techniques were used to separate the cells according to their density, which presumably corresponds to an age separation (see Discussion). These are respectively: the technique of Murphy [17] and a Percoll density gradient fractionation [20]. Both techniques are described in Materials and Methods. In both instances, erythrocytes were separated into three density (age) groups representing top (young), bottom (old) and middle (intermediate) fractions. The kinetics of outside-inside translocation of spin-labeled PS*, PE* and PC* were determined in these samples at 37 and 4°C, using the back exchange technique. The two techniques of separation gave qualitatively the same results, although the Percoll gradient technique gave more striking differences. Figs. 1 and 2 show representative data. Fig. 1 corresponds to an incubation at 37°C after Percoll gradient separation, while Fig. 2 displays data obtained at 4°C with samples separated by Murphy's method. With both techniques of separation and at both temperatures, the initial velocity of aminophospholipid translocation is reduced when going from top to middle and from middle to bottom fractions. Table I summarizes the results obtained at 37°C in a series of experiments involving the aminophospholipids. In spite of similar effects on the initial rates, the plateau levels of the two aminophospholipids are not equally sensitive to density separation. Only PE* steady-state distribution is reduced in the denser fraction, suggesting an increased fraction of PE on the outer monolayer. There is no significant variation of the plateau level for PS*. All these experiments were repeated several times. PS* results were very reproducible; with PE*, the plateau level of the lighter fraction, the reference level, obtained by extrapolation or by direct recording, varied slightly from one sample to another. However, there was always the same relative variation between the top, middle and bottom fractions, in particular the plateau reached by PE* in the bottom fraction was always $\approx 20\%$ lower than that of the top fraction. Note that PC* gave the same translocation rate for the three density separated fractions.

Role of intracellular ATP

The activity of the aminophospholipid translocase requires cytosolic ATP in the millimolar range [12,27]. In order to see if variations of cytosolic ATP level can explain the difference in translocase activity of 'aged' RBC, first the concentration of ATP was measured in the three fractions of packed cells. No significant difference of ATP concentration could be detected between the top and middle fractions in spite of the small difference of the initial velocities (v_i) of these two fractions. On the other hand, the ratio: $[ATP]_{bot}/[ATP]_{top}$ was 0.7 ± 0.1, suggesting that translocation rates might be altered because of differences in ATP level. However, in previous investigations we have shown

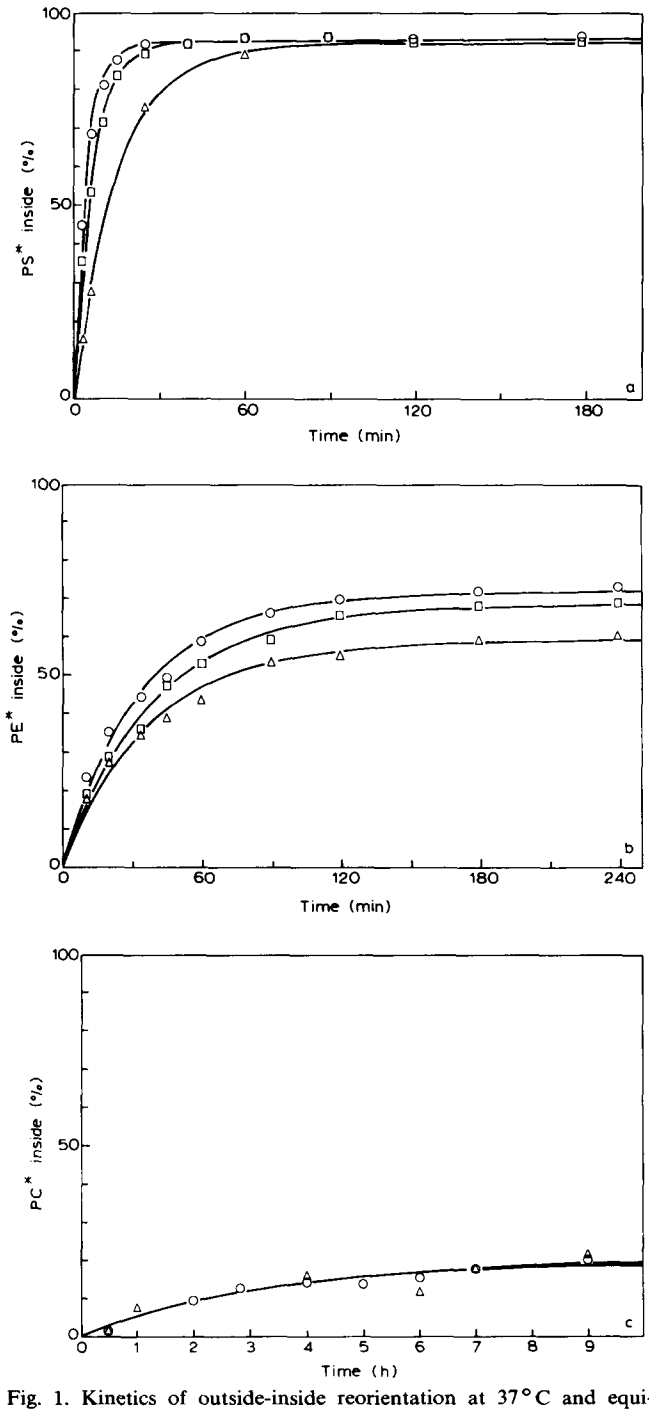

Fig. 1. Kinetics of outside-inside reorientation at 37°C and equilibrium distribution of spin-labeled phospholipids in different fractions of RBC separated by Percoll gradient. Respectively: (○) top, (□) middle and (△) bottom fractions. (a) Corresponds to PS*; (b) to PE*; (c) to PC*. Note the differences in scale of the abscissa. The curves shown here as well as in following figures, are fitted to an exponential function by least-square, non-linear regression.

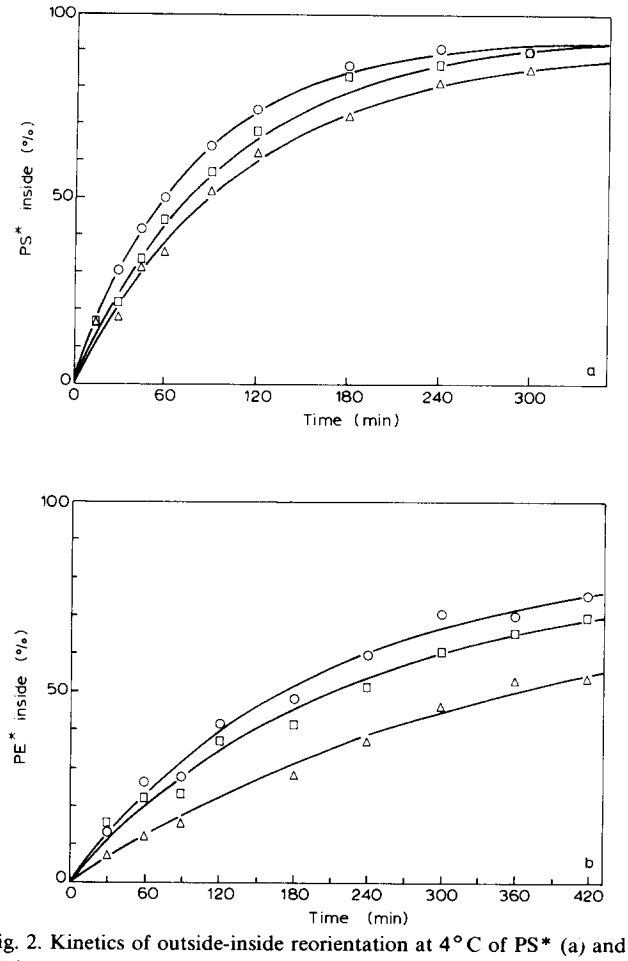

Fig. 2. Kinetics of outside-inside reorientation at 4°C of PS* (a) and PE* (b) in different fractions of RBC separated by the Murphy's method. (○) Top, (□) middle and (△) bottom fractions.

that a 30% variation of ATP concentration in the range 0.5–1 mM gives a non measurable variation of initial velocity (v_i) in ghosts resealed with a given concentration of ATP [27]. Furthermore attempts to correlate [ATP] and v_i for the various fractions in different experiments corresponding to PS* or PE* translocation gave no meaningful correlation (not shown). Finally, the difference between v_i of the top and bottom fractions, after Percoll separation, was maintained after ATP enrichment with glucose/inosine/adenosine buffer (not shown). In the latter experiments, the difference in ATP concentration between top and bottom fractions was cancelled. Thus, it appears that variations of ATP concentration alone cannot explain the difference in translocase activity between 'young' and 'old' cells.

TABLE I

Initial velocities of spin-label outside-inside translocation in the varing aged (density) fractions

Initial velocities in % per min at 37°C in RBC fractionated by the method of Murphy (A) or by Percoll gradient (B). Values are given ± standard error of estimate, n is the number of experiments.

Spin label	Method	Fraction:	Initial velocity (%/min) top	middle	bottom
PE*	A		1.92±0.07 $n=4$	1.71±0.10 $n=4$	1.65±0.11 $n=4$
	B		1.76±0.21 $n=3$	1.66±0.29 $n=3$	1.27±0.14 $n=3$
PS*	A		26.4±2.33 $n=3$	22.3±1.36 $n=3$	17.7±1.04 $n=3$
	B		24.9±2.09 $n=5$	18.9±2.22 $n=5$	12.10±1.58 $n=5$

Role of intracellular calcium

The physiological free calcium concentration in RBC is of the order of 20 nM [28]. An increased cytosolic calcium concentration would result in partial inhibition of the aminophospholipid translocase as shown in previous experiments carried out at 4°C [21]. Therefore we have set up to determine quantitatively the influence of cytosolic free calcium on the translocase activity at 37°C, by varying the intracellular calcium from ≈ 20 nM to 400 nM, a value which exceeds reported increase of calcium upon cell aging [29,30]. The latter experiments were carried out with PS* in the presence of inosine so as to maintain the level of intracellular ATP. We found no significant variation in plateau values and a progressive decrease in v_i to 75% of its initial value at intracellular free Ca^{2+} concentration of 400 nM. This is smaller tan the variations obtained after density separation (Table I). Thus, although the influence of Ca^{2+} cannot be eliminated, its role is not sufficient to explain the observed differences between top and bottom fractions.

ESR line shapes

In previous publications, we have shown that the spin-labeled lipids, used in the present work, are sensitive to the difference in environment between inner and outer monolayers of human RBC [14,31]. The ESR line shapes indicate a higher probe mobility in the inner monolayer. Recently we have demonstrated that this difference is due to the difference in phospholipid composition of the two monolayers [32]. A partial scrambling of the lipids should result in a partial homogenization of the environment. Yet, the difference in ESR line shape did not vanish when the probes were intercalated in red cells corresponding to the middle or the bottom fractions (not shown). Thus, the lipid redistribution which takes place in 'aged' cells is not sufficient to modify the asymmetrical physical properties which are detected by ESR spectroscopy.

Cell shape

Each fraction contained a majority of discocytes and a small fraction of echinocytes. No statistically signifi-

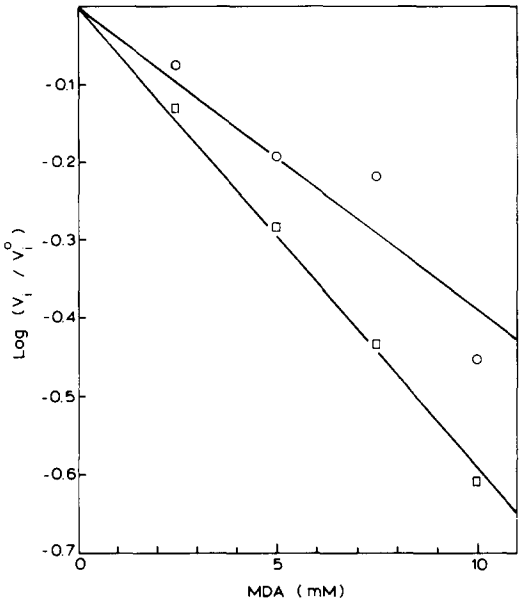

Fig. 3. Plots of the initial velocity of aminophospholipid translocation, v_i, versus MDA concentration, respectively, for (□) PS* and (○) PE*.

cant variations could be detected between top, middle and bottom fractions in agreement with Shukla and Hanahan [18].

Effect of MDA and H_2O_2 on the transverse mobility of phospholipids

Fresh RBC were treated with increasing concentrations of MDA and the outside-inside passage of spin-labeled phospholipids was monitored by ESR. The results obtained, respectively, with PS*, PE* and PC*, at 37° C, followed the general trend observed after density separation. The initial translocation velocity of the aminophospholipids (PS* and PE*) was diminished; PS* plateau remained constant while a decrease of PE* plateau was observed; PC* kinetics were barely affected, although a slight increase of initial velocity was noticeable. Fig. 3 shows that the initial velocity of PS* and PE* decrease exponentially with MDA concentration in the range 0–10 mM. With H_2O_2, we have tested the effect of 0, 0.5, 1, 2 and 4 mM. Incubation of RBC with 2 mM H_2O_2, for 1.5 h before translocation, resulted in a 30% decrease in the initial velocity of PE* translocation. However, incubation with higher concentrations of H_2O_2 (> 4 mM) did not give a reproducible decrease in v_i. This could be due to an artifact because at high concentration of H_2O_2 an irreversible loss of signal intensity was observed which results in an overestimation of v_i.

Discussion

The most commonly used method to isolate senescent RBC is that of density-dependent cell separation. Experimental evidence has been given to show that the normal red cell becomes progressively more dense as it circulates. A recent review by Clark [19] has shown the limits of this relationship, but concludes that, at present, there is no proven alternative to density-dependent methods for the isolated of senescent human red cells. Several functional impairments characterize dense red cells. See the review by Clark [19]: reduction in K^+ content which in turn affects the water content and the density; change in cell deformability; decline in metabolic activities; appearance of a 'senescent' antigen [9–11]. Alteration of the phospholipid asymmetry has been reported by Shukla and Hanahan [18]. According to these authors, 'old' cells have a higher proportion of PE on their outer monolayer than 'young' cells; PC distribution is not sensibly modified although PC flip-flop is accelerated; finally, no PS can be detected on the outer monolayer even in the most dense fraction. Similar results were reported by Tanaka and Schroit [3]. Thus, the published data on endogenous lipids fit very well with our data on the transport and equilibrium distribution of spin-labeled phospholipids, confirming the relevance of spin-label data. They suggest that for cells which are still in circulation, the lipid asymmetry is perturbed but not completely scrambled; although the mechanism of establishment and repair of lipid segregation is weakened, PS asymmetry is rigorous. The significance may be that the presence of PS on the outer monolayer is an immediate signal for cell elimination. If this is the case, it is not surprising that the heavy fraction, which corresponds to ≈ 5% of the cell population, contains a majority of cells which are capable of keeping PS from the outer monolayer. Indeed, as pointed out by Clark [19], even if most dense cells are senescent, cells that bear changes that will promote their removal are not likely to be plentiful, since in humans only ≈ 1% of the total cells is removed each day. However, our data can not be used to demonstrate that PS randomization is the trigger for cell elimination.

If PS asymmetry is important for RBC survival, the aminophospholipid translocase must function under safety conditions: a small decrease of the ATP concentration or of the number of active enzyme molecules must not modify the steady-state equilibrium distribution of PS. This is indeed the case. In previous publications, we have shown that the inhibition of the aminophospholipid translocation by NEM [13,16], by Ca^{2+} ions [13,21] or by thermal denaturation [27] yields a detectable change in the final distribution of the PS analogue only if the uptake rate decreases by more than 80% of its control value. In contrast the transbilayer distribution of the PE analogue is affected as soon as the translocation rate is slightly inhibited. The present article shows that similar observations are made after MDA or H_2O_2 treatment.

Thus, the modifications in lipid asymmetry which appear in the most dense RBC fraction, can be ex-

plained by a progressive inactivation of the aminophospholipid translocase. What causes such inactivation? In several interesting studies Jain has shown that the accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes [22,23]. In in vivo studies with rat erythrocytes, this author has shown that high doses of phenylhydrazine, an oxidant drug, produce externalization not only of PE but also of PS [33]. This perturbed lipid asymmetry could be due to an increased flip-flop of the phospholipids with an oxidized fatty acid chain. However, several laboratories have shown that the activity of the aminophospholipid translocase and, hence, the equilibrium distribution are not very sensitive to the nature of the fatty acid chain [16,34]. Thus, it is more likely that the aminophospholipid translocase itself is impaired by the products of the lipid peroxidation, such as MDA. Our experiments with MDA or H_2O_2 treated RBC reveal a partial inactivation of the enzyme by these products.

These experiments do not demonstrate that the translocase is the only protein modified. The present article shows that the impairment of the translocase is sufficient to explain the small modifications of the lipid asymmetry of 'old' cells. It has been reported that H_2O_2 caused the oxidation of about 60% of the sulfhydryl groups of RBC membranes [35]. Thus, the cytoskeleton proteins are also affected by aging and MDA treatment. Can this be the cause of the change in lipid asymmetry and translocation rates? We think that it is not the case because heat-induced RBC vesicles and heat-treated RBC are still capable to translocate spin-labeled aminophospholipids in spite of a complete denaturation of cytoskeleton proteins [27]. Furthermore Gudi et al. [36] have recently reported that in such spectrin denaturated RBC the asymmetry of endogenous lipids is maintained if the level of ATP is preserved, confirming that the membrane bilayer–skeleton interaction does not play an important role in maintaining transmembrane phospholipid asymmetry.

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