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Different behavior of ghost-linked acidic and neutral sialidases during human erythrocyte ageing

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Acidic and neutral sialidases (pH optimum 4.7 and 7.2, respectively) were assayed on human circulating erythrocytes during ageing. The assays were performed on intact erythrocytes and resealed erythrocyte ghost membranes. From young to senescent erythrocytes the acidic sialidase featured a 2.7-fold and 2.5-fold decrease in specific activity when measured on intact cells or resealed ghost membranes, whereas the neutral sialidase a 5-fold and 7-fold increase, respectively.

The Ca^{2+}-loading procedure was employed to mimic the vesiculation process occurring during erythrocyte ageing. Under these conditions the released vesicles displayed an elevated content of acidic sialidase, almost completely linked through a glycan phosphoinositide (GPI) anchor but no neutral sialidase activity, that was completely retained by remnant erythrocytes together with almost all the starting content of sialoglycoconjugates. The loss with vesiculation of acidic sialidase with a concomitant relative increase of neutral sialidase was more marked in young than senescent erythrocytes.

The data presented suggest that during ageing erythrocytes loose acidic sialidase, and get enriched in the neutral enzyme, the vesiculation process, possibly involving GPI-anchors-rich membrane microdomains, being likely responsible for these changes. The enhanced neutral sialidase activity might account for the sialic acid loss occurring during erythrocyte ageing.

Keywords: **human biochemistry, glycohydrolases, plasma membranes, glycoconjugates, blood cells**

Introduction

Circulating human erythrocytes, after an average life span of 120 days, are captured and endocytosed by macrophages of the reticular endothelial system. With ageing erythrocytes accumulate a series of molecular and behavioral modifications, primarily pertaining to the plasma membrane and its interactions with the cytoskeleton. These modifications flag red cells as "senescent" cells, which are then recognized and selectively removed from circulating blood [1–6]. Among the molecular events that concur to determine erythrocyte ageing, membrane vesiculating processes [7–11], autoimmune reactions [12–14], clustering of denatured hemoglobin and integral membrane proteins [15–17], protein damages from oxidative stress and advanced glycation end products [6,18,19], selective proteolysis of membrane components [20], and alteration of α-spectrin ubiquitination [21] may play relevant roles. Another peculiar event, which also occurs during ageing, in association or as a consequence of those mentioned before, is the loss of sialic acid by some erythrocyte membrane sialoglycoconjugates, particularly sialoglycoproteins, leading senescent erythrocytes to have a sialic acid content per cell 10–15% lower than that of young erythrocytes [22–27]. The loss of sialic acid from these sialoglycoconjugates can result from release of sialoglycopeptides promoted by proteolytic enzymes [20], and/or removal of sialic acid by the action of sialidase(s) [27–31]. Sialidases are known to be located in the membrane of human, as well as other mammalian, erythrocytes [32–37]. However, the involvement of surface sialidases of other cells (granulocytes, macrophages, endothelial cells) that are in contact with blood red cells during circulation [30], cannot be excluded.

The likelihood of sialidases to be involved in physiological erythrocyte desialosylation was considered to be problematic since known erythrocyte sialidases (of human, rat and rabbit origin) had an optimal pH in the acid range (4.2–4.7), displayed practically no activity at neutral pH [33–37], and were unable to affect endogenous sialoglycoproteins [38].

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Recently, we reported the presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH, and capable to hydrolyze endogenous sialoglycoproteins [38].

On these bases, and with the aim to define the possible involvement of sialidase(s) in human erythrocyte ageing, we decided: (a) to study the behaviour of the "acidic" and "neutral" sialidases during red cell physiological ageing, using as the source venous blood from healthy normal volunteers and preparing young and old erythrocytes by the Percoll discontinuous density gradient centrifugation method; and (b) to assess the effect on the two sialidases of membrane vesiculation, one of the most remarkable processes leading erythrocytes to senescence; as a model for vesiculation we employed the Ca^{2+} loading approach [7].

Methods

Chemicals

Commercial chemicals were of the highest grade available. Recombinant phosphatidylinositol specific phospholipase C (PIPLC) from *Bacillus Thuringiensis* (2000 U/mg) was purchased from Oxford Glyco System (Abingdon, U.K.); 4-methylumbelliferone (MU), 4-methylumbelliferyl -α-N-acetyl-D- neuraminic acid (MU-NeuAc), N-Acetyl-D-neuraminic Acid (NeuAc), creatine hydrate, crystalline bovine serum albumin, N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), Percoll and Ca^{2+}-ionophore A23187 from Sigma Chemical Co. (St. Louis, MO, USA). Dowex 2×8 resin (200 to 400 mesh), prepared in acetate form according to Svennerholm [39] was supplied by Bio-Rad Laboratories (Richmond, VA, U.S.A). Water was doubly distilled in a glass apparatus and used to prepare the different solutions. Ganglioside GD1a and its tritiated form ([^{3}H]GD1a) were prepared as described previously [38].

Blood collection and isolation of red cells from blood samples

Following informed consent, heparinized human blood samples (3–6 ml) from healthy adult individuals (male and female blood donors; age ranging from 25 to 40 years) were processed within 1 hour from collection. Erythrocytes were separated from leukocytes and platelets by filtering through a column of α-cellulose and microcrystalline cellulose (2:1 by weight), according to the method of Beutler et al. [40], in HEPES buffered isotonic saline: 133 mM NaCl, 4.5 mM KCl, 10 mM HEPES, pH 7.4. The hematocrit of the filtered blood was adjusted to approximately 30% and the suspensions stored at 4°C until used (within 1 h). For most experiments blood pools from 5–6 donors were employed; in some cases erythrocytes were prepared from the blood of single donors.

Fractionation of erythrocytes according to their age: young, average-aged and old (senescent) erythrocytes

Human erythrocytes were fractionated into young, average-aged and old erythrocytes by the Percoll discontinuous density gradient/centrifugation method as described by Salvo et al. [41], and modified by Mosca et al. [42]. Briefly, 4 ml of filtered blood (hematocrit 30%, containing $2–2.4 \times 10^{10}$ cells) in 10 mM HEPES buffer, pH 7.4, containing 133 mM NaCl, 4.5 mM KCl, were layered on top of the discontinuous gradient (from the bottom of 36 ml tube: 1 ml, 80%; 8 ml, 74%; 8 ml, 70%; 8 ml, 66%; and 4 ml 60% Percoll). Centrifugation was carried out in a swing-out rotor at $2000 \times g$ for 15 min at 20°C. The centrifuge was slowly decelerated over 3 min to prevent disturbance of the gradient. Plasma (about 1.5 ml), which remained at the top of the gradient, was removed and the cell fractions, visually recognisable at the interfaces, were collected by aspiration from the top of the gradient using a Pasteur pipette attached to a peristalting pump and manoeuvred manually over the liquid surface (about 26 fractions, usually 1.2 ml per tube).

Each fraction was characterised with respect to cell age according to Fehr and Knob [43], by measuring MCV with a Cell Counter (Model Z_{BI}, Coulter Electronics, Hialeah, FL), and creatine by the method of Griffiths [44]. In the Percoll gradient, the fractions containing young cells were stratified at the top of the gradient (layer over 66% Percoll), the average-aged cells layered over 70% and 74% Percoll, and the fractions containing the senescent cells layered over 80% Percoll. The fractions collected over each layer were pooled, centrifuged at $2500 \times g$ for 20 min, and washed three times with HEPES buffered isotonic saline at 4°C, in order to remove Percoll. The final pellets were stored at 4°C (no longer than 1 h), and homogeneously suspended with HEPES buffered isotonic saline solution immediately before use.

Preparation of unsealed ghost membranes, resealed ghost membranes and inside-out vesicles from different erythrocyte populations

Unsealed ghost membranes from young, average-aged and old erythrocytes were prepared at 4°C according to the method of Steck and Kant [45] which employs an hypotonic treatment (from 5.0 to 1.25 mM PBS, pH 7.2). Resealed ghost membranes were prepared according to Steck et al. [46] and following the modifications introduced by Venerando et al. (38), by simply dispersing unsealed ghost membranes (obtained from different erythrocyte populations) with 40 vol. of 5 mM PBS, 0.15 M NaCl buffer, pH 8.0 and allowing the dispersion to stand at 37°C for 40 minutes. As previously reported (38) this treatment proved to unmask the activity of neutral sialidase. Inside-out vesicles were prepared as described by Steck et al. [46] and lysed as reported by Venerando et al. [38]. Assessment of resealing of unsealed ghost membranes and purity of inside-out vesicles, as well as verification of efficacy of inside-out vesicles lysis were

performed by determining the activity of acetylcholinesterase and NADH-cythocrome C-oxidoreductase.

Erythrocyte vesiculation treatment: preparation and isolation of "vesicles"

Six ml of filtered blood (hematocrit 30%) or 4 ml of young or senescent erythrocyte populations both containing $3–3.4 \times 10^{10}$ cells, were resuspended with 10 ml of 10 mM HEPES isotonic buffer, pH 7.4 and incubated at 37°C in the presence of 0.8 mM $CaCl_2$ and 8 μM of Ca^{2+}-ionophore A23187, essentially according to Allan et al. [47]. After 1 h, the reaction was stopped with 4 ml of 60 mM EGTA solution (in order to remove Ca^{2+} ions), erythrocytes precipitated by centrifugation at 1000 × g for 5 min, and the cloudy supernatant (containing "vesicles") collected. "Vesicles" were purified from cellular debris, contaminating intact erythrocytes, and remnant erythrocytes by centrifugation in a self-forming Percoll gradient, according to the procedure described by Iida et al. [48]. The collected "vesicles" and remnant erythrocytes were stored at 4°C (no longer than 1 h), and homogeneously suspended with HEPES buffered isotonic saline solution immediately before use.

Release of sialidase from young and senescent erythrocyte resealed ghost membranes and "vesicles" by treatment with PIPLC

Resealed ghost membranes, or "vesicles", obtained from young and senescent erythrocytes were treated with PIPLC according to Venerando et al. [38]. Briefly, the membranes, or "vesicles", specimens (approximately 0.8 mg, as protein) were washed with 2.0 ml of 25 mM Tris-acid acetic buffer 0.15 M NaCl, pH 7.4, centrifuged (10,000 × g for 15 min) and the pellets dissolved in 0.7 ml of the same buffer. An aliquot of PIPLC (protein/enzyme units ratio, 7 mg/0.5 U) was added and the mixtures incubated at 37°C for 30 min. At the end of the incubation, an additional aliquot of PIPLC (equal to that used in the first treatment) was added to the same incubation mixtures, and the incubation allowed to continue for the same time employed in the first treatment. The addition of PIPLC and prolongation of incubation were repeated twice again. At the end of the last incubation, the mixtures were centrifuged at 28,000 × g for 20 min, and the supernatants centrifuged at 4°C (150,000 × g for 10 min). The final supernatants and pellets were analysed. In all the experiments a blank incubated without PIPLC was run concomitantly.

Action of young and senescent erythrocyte membrane-bound sialidases on endogenous substrates

Incubation mixtures containing: 0.5 ml of young or senescent erythrocyte resealed ghost membranes (2.5 mg, as protein), 0.1 ml of 0.5 M citric acid-sodium phosphate buffer at 4.7 or 7.2 pH (corresponding to the optimal pH of the acidic and neutral sialidase, respectively) and 0.4 ml of redistilled water were incubated at 37°C for 18 hours with gentle shaking. At the end, the mixtures were brought to pH 2.5 by slow dropwise addition of 1 M HCl and then centrifuged at 4°C for 10 min at 150,000 × g. The clear supernatants were quantitatively transferred on the top of Dowex 2 × 8 columns (0.25 × 0.5 cm, in acetate form) and free sialic acid eluted and determined according to Caimi et al. [49]. The blank mixtures had the same composition and treatment as the assay mixtures, the only difference being that the young and senescent erythrocyte resealed ghost preparations were previously inactivated by immersion in a boiled water bath for 10 min.

Determination of total, ganglioside (or lipid)-bound and glycoprotein-bound sialic acid content in young and senescent erythrocyte resealed ghost membranes and "vesicles"

Total sialic acid content was determined by the chromatographic micro procedure of Caimi et al. [49], on samples digested for 90 min in 0.05 M sulphuric acid at 80°C, from which insoluble material was removed by centrifugation. Ganglioside-bound and glycoprotein-bound sialic acid were determined according to Tettamanti et al. [50].

Sialidase assays

The assay for membrane-bound and PIPLC-released sialidases, in the various preparations obtained from young, average-aged and old erythrocytes, employed the fluorimetric and radiochemical methods described in a previous paper [38], using saturating substrate concentrations. The substrate used for the fluorimetric assay was MU-NeuAc and [^{3}H]GD1a for the radiochemical one. The (assessed) optimal pH used was 4.7 and 7.2 for the acidic and neutral sialidase, respectively. The assay for sialidase on intact erythrocytes (of different age) or "vesicles" (both containing high hemoglobin amounts) was performed with the radiochemical method described above, which was adapted and optimalized for this purpose. The assay mixtures, containing, in a final volume of 0.1 ml, the erythrocyte suspension (10–20 μl, corresponding to 51–102 nM of phospholipid phosphorus), or the "vesicles" suspension (20–40 μl corresponding to 40–80 nmol of phospholipid phosphorus) in 0.15 M NaCl, 150 nM [^{3}H]GD1a (carrying 2.5×10^5 dpm), 50 mM citric acid-sodium phosphate buffer, 0.15 M NaCl at pH 4.7 or 7.2, were incubated at 37°C for 2 hours under gentle shaking. Incubations were terminated by immersion in an ice-water bath. The refrigerated mixtures were centrifuged (1000 × g for 5 min), and an aliquot of each supernatant was withdrawn for hemoglobin determination (as a marker of hemolysis), while the remainder portion was dialysed against 100 vol. of re-distilled water (four changes) for one night, in order to remove phosphate. The materials were lyophilised and the residues suspended with chloroform/methanol, 2:1, by volume, in order to extract lipids, and centrifuged. Blank mixtures were prepared with boiled (10 min) erythrocyte preparations. The separation and radiochromatoscanning determination of radiolabeled compounds,

recovered in the supernatants, were accomplished as described [38]. Enzyme activities were expressed as units (U), that is the amount of enzyme that liberates 1 μM of product min/mg protein, or μM phospholipid phosphorus at 37°C, under optimal conditions.

Other methods

Protein content was determined by the method of Lowry et al. [51], using crystalline bovine serum albumin as the standard; when Tris buffer was present, the Coomassie Brillant Blue method [52] was used. Acetylcholinesterase (Ach-esterase) activity was determined by the method of Ellman et al. [53], and NADH-cytochrome C oxidoreductase (NADH-Cyt C ox/red) by the method reported by Steck and Kant [45]. Total phospholipids were extracted according to Ladisch and Gillard [54] and determined as bound phosphorus (P) by the method of Barlett [55]. Hemoglobin was assayed as reported by Taguchi et al. [56].

Statistical analysis

Since no significant differences from the normal distribution were observed, parametric analysis techniques were used, and the standard deviation of means calculated [57].

Results

Behaviour of acidic and neutral sialidases during ageing of human erythrocytes

The study of acidic and neutral sialidase behaviour during ageing was accomplished on preparations of young, average-aged and senescent erythrocytes separated on a Percoll discontinuous density gradient as described in the experimental section. After centrifugation on the gradient, four visible cell layers were obtained, as expected [41,42]: one (the smallest one) at 66%, two (the largest ones) at 70% and 74%, respectively, and one at 80% Percoll. On the basis of creatine content, which markedly decreased from the first to the fourth cell layer (from 5 to 0.2 mg for 10^9 cells) and Ach-esterase content (which also decreased in parallel) and following reported criteria [41,42,58], the fractions from the first layer with a creatine content ranging from 5 to 3 mg for 10^9 cells were pooled and constituted the "young erythrocytes" population, and the fractions from the fourth layer with a creatine content between 0.6 and 0.2 mg for 10^9 cells were also pooled and constituted the "old or senescent erythrocytes" population. The fractions from the second and third layers, carrying 1–2 mg creatine for 10^9 cells, were pooled together and considered as the "average-aged erythrocytes".

As shown in Figure 1A, the unsealed ghost membranes obtained from young to senescent erythrocytes carried a seemingly unique sialidase activity (assayed on MU-NeuAc) with optimal pH 4.3–4.7, with a trace of residual activity extending through the neutral pH range. The specific activity

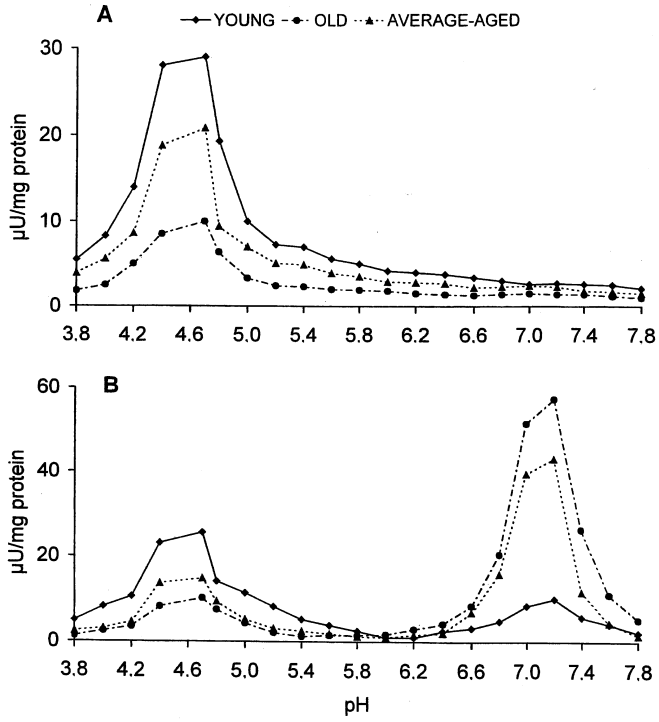

Figure 1. Effect of pH on the sialidase activity contained in the unsealed (A) and resealed (B) ghost membranes obtained from young, average-aged and old erythrocytes.

of the acidic enzyme was higher in young erythrocytes and diminished gradually to a one third value in old cells. Conversely, the corresponding resealed ghost membranes (Figure 1B) carried, besides the acidic one, also a sialidase activity with a pH optimum 7.2 (neutral sialidase), whose specific activity markedly increased from young to old erythrocytes. Therefore, the procedure used to reseal ghost membranes (dispersion with 40 volumes at 5 μM phosphate, 0.15 M NaCl buffer, pH 8.0, incubation at 37°C for 40 min) was capable of unmasking neutral sialidase activity, as already observed in unfractionated erythrocyte [38].

In Table 1 the values of specific activity of acidic and neutral sialidases (referred to both mg protein and μM phospholipid phosphorus) determined in young and old erythrocytes from 25 donors are reported. From young to senescent erythrocytes the specific activity of the neutral sialidase underwent a 7.0-fold (referred to mg protein) and 8.6-fold (referred to phospholipid phosphorus) increase, whereas that of the acidic enzyme a 2.5-fold (referred to mg protein) and 2.0-fold (referred to phospholipid phosphorus) decrease. Thus the acidic/neutral sialidase ratio appears to undergo a shift with age from 3.1 to 0.16 and 3.1 to 0.17, with reference to mg protein and μM phospholipid phosphorus, respectively, with a relative enrichment of the neutral enzyme over the acidic one of 19-fold and 18-fold, referred to the two parameters, respectively. The S.D. values of the sialidase specific activities were generally lower than 10% of the mean

Table 1. Specific activity (referred to mg/protein or μM phospholipid phosphorus) and substrate affinity (expressed as apparent km) of acidic and neutral sialidases present in resealed ghost membranes obtained from young, average-aged and old human erythrocytes. Sialidase was assayed using MU-NeuAc as substrate

	Acidic sialidase specific activity			*Neutral sialidase specific activity*		
Erythrocyte (age)	*(μU/mg protein)*	*(μU/μM P)*	*Km (μM)*	*(μU/ mg protein)*	*(μU/μM P)*	*Km (μM)*
Young	25.6 ± 2.1	22.8 ± 2.1	25.5	8.2 ± 0.7	7.3 ± 0.6	5.0
Average-aged	14.8 ± 1.3	15.5 ± 1.4	26.0	43.2 ± 4.0	43.1 ± 3.9	4.7
Old	10.1 ± 1.2	11.2 ± 1.3	28.3	57.6 ± 4.9	63.8 ± 5.1	4.5

The determination of km values was accomplished using resealed ghost membranes obtained from a blood pool from 5–6 donors. The determination of acidic and neutral sialidase specific activity was carried out on the blood of 25 different donors, each determination being accomplished in triplicate. The data exposed are means ± D.S. values. P: phospholipid phosphorus.

values, with the exception of the acidic sialidase in old erythrocytes, where they reached about 12%, all this indicating a relatively low degree of biological variability. As shown also in Table 1 the apparent Km values of the two enzymes (toward MU-NeuAc) remained practically unchanged during ageing (25.5–28.3 μM for the acidic enzyme; 5.0–4.5 μM for the neutral one). The same behaviour with age of the two sialidases was exhibited with gangliosidic substrates (GM3, GD1a) (data not shown).

The data exposed in Table 2 describe the side topology of the acidic and neutral sialidases in human erythrocyte membrane during ageing. As shown, Ach-esterase, which is known as a typical enzyme of the external membrane surface, expectedly [38] was fairly measurable throughout the ageing process on unsealed and resealed ghosts, present in trace amounts in inside-out vesicles and easily measurable in lysed inside-out vesicles (data not shown). Moreover, NADH-CytC-Ox/Red, which is known to expose its active site on the internal membrane surface, during the ageing process appeared, as expected, to be much more abundant in inside-out vesicles than in resealed ghost membranes and lysed inside-out vesicles (data not shown). The acidic sialidase, regardless of the erythrocyte age, was easily measured in unsealed ghost membranes and resealed ghost membranes, but could not be detected in inside-out vesicles (reappearing after their lysis), indicating a permanent location with ageing in the external membrane surface, as previously shown in a preparation of unfractionated erythrocytes [38]. The activity of the neutral sialidase, fully expressed in the resealed ghost membranes during ageing, was unmeasurable in unsealed ghost membranes (where it is essentially masked) [38], and undetectable in inside-out vesicles, suggesting a location in the

Table 2. Acidic and neutral sialidases, Ach-esterase, NADH-Cyt C Ox/Red content in unsealed ghost membranes, resealed ghost membranes and inside-out vesicles obtained from human erythrocytes of different age

	Sialidase specific activity μU/mg protein		*Ach-Esterase specific activity U/mg protein*	*NADH-Cyt.C Ox/Red specific activity mU/mg protein*
Erythrocyte (age)	*Acidic*	*Neutral*		
Young				
Unsealed ghost membranes	28.0 ± 1.7	2.7 ± 0.1	3.2 ± 0.1	20.5 ± 0.6
Resealed membranes	25.8 ± 0.9	8.3 ± 0.5	2.9 ± 0.1	1.0 ± 0.06
Inside-out vesicles	n.d.	n.d.	0.10 ± 0.008	21.0 ± 0.7
Average-aged				
Unsealed ghost membranes	19.6 ± 0.9	2.3 ± 0.1	2.8 ± 0.1	17.9 ± 0.4
Resealed membranes	14.7 ± 0.7	42.9 ± 1.3	2.6 ± 0.1	1.3 ± 0.02
Inside-out vesicles	n.d.	n.d.	0.07 ± 0.005	18.8 ± 0.3
Old				
Unsealed ghost membranes	9.3 ± 0.5	1.4 ± 0.05	1.9 ± 0.1	15.5 ± 0.30
Resealed membranes	10.2 ± 0.4	57.9 ± 1.5	1.8 ± 0.05	1.1 ± 0.03
Inside-out vesicles	n.d.	n.d.	0.04 ± 0.003	16.0 ± 0.4

For details see the Experimental Section. The data are the mean values of five experiments ± SD values, using in each experiment a blood pool from 5–6 donors. n.d., not detectable.

external membrane surface too. The diminution of sialidase specific activity at pH 7.2 in unsealed ghost membranes paralleling the concurrent decrease of activity at pH 4.7 further support the notion that the sialidase activity at pH 7.2, recorded in these preparations, is mainly attributable to residual activity of the acidic sialidase.

As shown in Table 3, and as expected, the total content of bound sialic acid in resealed ghost membranes was substantially lower in old than in young erythrocytes (41.2 μg against 54.6 μg/mg protein), with half the ganglioside-bound sialic (1.7 against 3.5 μg/mg protein) and a diminution of the glycoprotein-bound sialic acid from 48.0 μg to 37.0 μg/mg protein from young to old erythrocytes. However, the most dramatic drop in sialic acid content was observed not from young to average-aged, but from average-aged to senescent erythrocytes. In both young and old erythrocyte ghost resealed membranes (Table 4), the acidic sialidase was able to affect endogenous gangliosides but not sialoglycoproteins, whereas the neutral enzyme hydrolyzed both substrates, a feature already observed on unfractionated erythrocytes [38]. The percentage of hydrolysis was proportionately higher in young than old erythrocytes, at both the pH optima of the two sialidases (4.7 and 7.2): for gangliosides 81.6% versus 62.6% at pH 4.7, and 72% versus 30% at pH 7.2, respectively, and for sialoglycoproteins 16.4% versus 3.0% at pH 7.2.

Exhaustive treatment of the resealed ghost membranes with PIPLC (see Table 5) from young and senescent erythrocytes caused a marked release of the acidic sialidase, but had no

Table 3. Total-bound, ganglioside-bound and glycoprotein-bound sialic acid present in the resealed ghost membranes obtained from human erythrocytes of different age

	Total-bound Sialic acid		*Ganglioside-bound Sialic acid*		*Glycoprotein-bound Sialic acid*		*Recovery*	
Erythrocyte (age)	*μg/mg protein*	*%*	*μg/mg protein*	*%*	*μg/mg protein*	*%*	*Total*	*%*
Young	54.6 ± 1.6	100	3.5 ± 0.4	6.5	48.0 ± 2.4	88.0	51.5	95.2
Average-aged	54.0 ± 2.2	100	3.2 ± 0.3	6.0	47.8 ± 1.44	88.5	51.0	94.5
Old	41.2 ± 1.2	100	1.7 ± 0.1	4.0	37.1 ± 1.30	90.0	38.7	94.1

The data are the mean values, ± SD, of six experiments, using in each case a pool of blood obtained from 5–6 donors. Each experiment was accomplished in duplicate. For details see Experimental Section.

Table 4. Action of acidic and neutral sialidases present in resealed ghosts membranes of young and old erythrocytes on endogenous substrates (glycolipids and glycoproteins)

		After Incubation	
	Before Incubation	*pH 4.7*	*pH 7.2*
Young			
Ganglioside-bound sialic acid	3.54 ± 0.36	0.65 ± 0.02	0.99 ± 0.06
Released sialic acid (%)	0	81.6	72
Protein-bound sialic acid	48.0 ± 2.44	48.1 ± 2.16	40.1 ± 1.2
Released sialic acid (%)	0	0	16.4
Total bound sialic acid	54.6 ± 1.64	51.7 ± 4.12	44.1 ± 1.32
Released sialic acid (%)	0	5.3	19.1
Old			
Ganglioside-bound sialic acid	1.7 ± 0.11	0.62 ± 0.03	1.16 ± 0.11
Released sialic acid (%)	0	62.6	30
Protein-bound sialic acid	37.1 ± 1.3	37.0 ± 0.92	36.0 ± 1.3
Released sialic acid (%)	0	0	3.0
Total bound sialic acid	41.2 ± 1.23	40.1 ± 3.4	39.5 ± 1.0
Released sialic acid (%)	0	2.54	4.0

The Resealed Ghost membranes were incubated at pH 4.7 and pH 7.2 as specified in the Experimental Section, and ganglioside-bound, glycoprotein-bound and total sialic acid determined before and after incubation.
Data are expressed as μg of total-bound, ganglioside-bound and glycoprotein-bound sialic acid per mg protein. The data are the mean values of six experiments ± SD values. In each experiment, performed in duplicate, a pool of blood obtained from 5–6 donors was employed.

Table 5. Release of acidic and neutral sialidases and ach-esterase from the resealed ghost membranes of young and old erythrocytes by exhaustive treatment with PIPLC from *Bacillus thuringensis*

	Protein		*Acidic Sialidase*			*Neutral Sialidase*			*Ach-Esterase*		
	Total (mg)	*Recovery (%)*	*Specific Activity (μU/mg prot.)*	*Total Activity (μU)*	*Recovery (%)*	*Specific Activity (μU/mg prot.)*	*Total Activity (μU)*	*Recovery (%)*	*Specific Activity (U/mg prot.)*	*Total Activity (U)*	*Recovery (%)*
Young											
Starting erythrocyte preparation	0.80	100	25.6 ± 0.9	20.48	100	8.20 ± 0.48	6.56	100	2.90 ± 0.10	2.32	100
Supernatant	0.10	12.5	181.0 ± 6.8	18.10	88.4	n.d.	n.d.		0.53 ± 0.01	0.15	6.8
Pellet	0.66	82.5	2.0 ± 0.08	1.32	6.4	9.43 ± 0.78	6.23	95	2.67 ± 0.08	1.76	76.1
Total recovery	0.76	95.0	–	19.42	94.8	–	6.23	95	–	1.91	82.3
Old											
Starting erythrocyte preparation	0.82	100	10.1 ± 0.4	8.28	100	57.6 ± 2.9	47.23	100	1.80 ± 0.07	1.48	100
Supernatant	0.11	13.4	50.1 ± 4.0	5.51	66.6	n.d.	n.d.		0.54 ± 0.02	0.06	4.2
Pellet	0.65	79.3	4.0 ± 0.14	2.60	31.4	67.6 ± 4.7	43.9	93	1.89 ± 0.08	1.23	83.3
Total recovery	0.76	92.7	–	8.11	98.0	–	43.9	93	–	1.29	87.2

The data are the mean values, ± SD, of five experiments each of them using a blood pool from 5–6 donors. n.d., not detectable; prot = protein. For details see Experimental Section.

influence on the neutral enzyme, a behaviour that was previously observed in preparations of unfractionated erythrocytes [38]. However, the efficacy of treatment was higher in young than senescent erythrocytes, the degree of enzyme solubilization reaching 88.4% in the former and 66.6% in the latter erythrocytes. This behaviour was paralleled by that of Ach-esterase, although with a much lower extent of release, as expected.

In order to explore whether the age-related enrichment of the neutral sialidase could be observed by directly assaying the enzyme in intact erythrocytes of different age, we incubated young, average-aged and old erythrocytes under the conditions previously set up [38], using as substrate [^{3}H] GD1a. As shown in Table 6, the neutral sialidase specific activity (referred to phospholipid phosphorus) underwent a 5-fold increase from young to senescent erythrocytes, with a much greater increase from average-aged to senescent erythrocytes than from young to average aged-cells. Parallelly, the specific activity of the acidic sialidase presented a 2.7-fold decrease from young to old erythrocyte. The measured values of specific activity of both sialidases (referred to μM phospholipid phosphorus) were lower (from 30% to 70%) than those measured on resealed ghost membranes.

Table 6. Direct assay of acidic and neutral sialidase activity on human unfractionated, young, average-aged, and old erythrocytes

Erythrocyte	*Acidic Sialidase Activity (μU/μM P)*	*Neutral Sialidase Activity (μU/μM P)*
Unfractionated	14.30 ± 0.70	4.56 ± 0.23
Young	17.32 ± 1.12	3.58 ± 0.28
Average-aged	11.90 ± 0.75	5.33 ± 0.24
Old	5.31 ± 0.34	18.50 ± 1.50

Assays were performed at pH 4.7 and 7.2 using 10–20 μl erythrocyte suspension corresponding to 51–102 nmol of phospholipid phosphorus. Acid and neutral sialidases were assayed by the radiochemical method with ^{3}[H] GD1a as substrate. At the end of incubation an aliquot of the mixture was withdrawn, centrifuged, and hemoglobin content determined in the supernatant. The degree of hemolysis was in the range 1.80–1.92%. The data are the mean values of five experiments, ± S.D. values, each of them using a blood pool from 5–6 donors. Each experiment was carried out in triplicate. P = phospholipid phosphorus.

In vitro membrane vesiculation of erythrocytes of different age: behaviour of acidic and neutral sialidases

As a study model of erythrocyte membrane vesiculation we used the treatment with 0.8 mM $CaCl_2$ and 8 μM Ca^{2+}-ionophore A23187, followed by centrifugation on a discontinuous Percoll density gradient, as described in the experimental section. Owing to the presence of hemoglobin in the formed vesicles the radiochemical assay method for sialidases was employed (with [^{3}H] GD1a as substrate) and the enzyme activity expressed per μM phospholipid phosphorus. Using unfractionated erythrocytes (see Table 7) acidic sialidase was present in the formed "vesicles" where the enzyme specific activity appeared to be 3.5-fold enriched as compared to the starting erythrocytes; conversely, no detectable neutral sialidase activity occurred in "vesicles". Upon exhaustive treatment with PIPLC, 93.5% of the acidic sialidase bound to "vesicles" was released and recovered in the supernatant obtained after centrifugation. Under the same conditions 83.2% of the enzyme, present in unfractionated erythrocytes, was liberated. Both glycolipid-bound and glycoprotein-bound sialic acid were present in "vesicles", but with a specific concentration markedly lower (about 1.3%) than that of the starting erythrocytes. Also using young and senescent erythrocytes (Table 8) the formed vesicles carried acidic sialidase but not the neutral sialidase. Noteworthy, the specific activity of acidic sialidase in "vesicles" versus that of the starting erythrocytes raised from about 2-fold in young erythrocytes to about 17-fold in senescent erythrocytes.

Table 7. Acidic and neutral sialidase activity and releasability by PIPLC treatment, and bound sialic acid (total, ganglioside-bound and protein-bound) in "vesicles" produced by "Ca^{2+} loading" of unfractionated human erythrocytes

	Sialidase						
	Activity (μU/μM P)		*Releasability by PIPLC treatment (%)*		*Sialic Acid*		
	Acidic	*Neutral*	*Acidic*	*Neutral*	*Total-bound*	*Protein-bound*	*Ganglioside-bound*
Unfractionated Erythrocytes	14.3 ± 0.7	4.2 ± 0.21	83.2	0	1785.7 ± 135.7	1571.4 ± 122	209.7 ± 12.8
Vesicles	47.0 ± 2.1	n.d.	93.5	0	23.4 ± 1.21	20.7 ± 0.93	2.6 ± 0.12

Vesicles were prepared by treatment of human erythrocytes with 0.8 mM $CaCl_2$ and 8 μM Ca^{2+} ionophore A23187 followed by Percoll density gradient centrifugation, as described in Experimental Section. The acidic and neutral sialidases were determined by the radiochemical assay method with ^{3}[H] ganglioside GD1a as substrates PIPLC treatment was accomplished on the resealed ghost membranes, obtained from unfractionated erythrocyte as reported in the Experimental Section. Total, protein-bound and ganglioside-bound sialic acid were determined as reported in the Experimental Section. The presence of A23187, assayed up to 2 μM, was proved not to influence the activity of sialidases. The data are the mean values of five experiments ± S.D. values, each of them using a blood pool from 5–6 donors. Each experiment was carried out in duplicate. P = phospholipid phosphorus.

Table 8. Acidic and neutral sialidases in the vesicles obtained from young and old human erythrocytes. Vesicles were prepared by the CA^{2+}/ionophore A 23187/percoll density gradient centrifugation method and sialidases determined by the radiochemical method with [^{3}H]GD1a as substrate

	Sialidase Activity (μU/μM P)	
Erythrocyte (Age)	*Acidic sialidase*	*Neutral sialidase*
Young		
Starting erythrocytes	17.32 ± 1.12	3.58 ± 0.28
Vesicles	31.8 ± 2.38	n.d.
Remnants	20.5 ± 1.33	18.30 ± 1.50
Debris	n.d.	n.d.
Senescent		
Starting erythrocytes	5.3 ± 0.34	18.50 ± 1.50
Vesicles	89.3 ± 5.35	n.d.
Remnants	4.8 ± 0.29	20.0 ± 1.68
Debris	n.d.	n.d.

The data reported are the mean values, ± S.D., of five experiments, each of them using a blood pool from 5–6 donors.
n.d., not detectable; P = phospholipid phosphorus.

Discussion

Behaviour of acidic and neutral sialidases during ageing of human erythrocytes

In a recent paper [38] we described the occurrence in human erythrocyte plasma membrane of a novel sialidase, optimally acting at neutral pH, able to split sialic acid from sialoglycoproteins—the predominant sialoglycoconjugates in erythrocytes—and fully resistant to the action of PIPLC. This sialidase differed from the previously known erythrocyte sialidase [34–36], which has an acidic optimal pH, does not affect sialoglycoproteins, and is sensitive to PIPLC treatment. The present report shows that erythrocyte ageing is accompanied by a marked enrichment of the neutral sialidase, in terms of specific activity, with concomitant decrease of the acidic enzyme. In fact from young to senescent erythrocytes the specific activity of the neutral sialidase over that of the acidic enzyme underwent a 19-fold increase when referred to the protein content and a 18-fold increase with reference to the phospholipid content. Since the two enzymes, as proteins, cannot be synthesized in mature, circulating, erythrocytes but only possibly degraded by proteolysis, the remarkable changes in their specific activity are likely attributable to the profound compositional modifications occurring at the erythrocyte plasma membrane during ageing [7–11,15–17,20–22]. Depending on the fact that during ageing the two sialidases are either released, as well as other protein and phospholipid components [59–61] from, or retained by the plasma membrane, their specific activity may relatively decrease or increase. It is worth noting that both the remarkable increase with ageing of the neutral sialidase specific activity and the decrease of that of the acidic enzyme were equally featured by all the 25 blood donors, whom were analyzed, with a low interindividual variability. Therefore, the relative enrichment of the neutral over the acidic sialidase specific activity (18/19-fold) can be considered a good age parameter for red cells, in addition to others [43,62]. This aspect would deserve further attention.

The properties of the two enzymes (optimal pH; Km values toward MU-NeuAc and gangliosides; location on the outer surface of the plasma membrane; susceptibility of the acidic enzyme to PIPLC) remained unchanged during erythrocyte ageing, indicating that the enzyme molecules per se did not undergo substantial changes during the ageing process. However, some relevant differences in their behavior were recorded. One difference is the higher degree of endogenous sialoglycoconjugates hydrolysis by the acidic and neutral sialidases in the young than senescent erythrocytes. Particularly, the hydrolysis of sialoglycoproteins promoted by the neutral sialidase was much greater in young than old erythrocytes (16.4% against 3.0%). This reflects either a different availability of endogenous sialoglycoproteins to the enzyme inside the membrane, and/or a selection of individual sialoglycoproteins (or sialosylated sites on the same glycoconjugate) by the enzyme, the more susceptible ones being affected earlier. The latter interpretation may be favored by the observation, reported here (see Table 3), that the major drop in total bound-sialic acid content during ageing occurred from average-aged to old erythrocytes and not from young to average-aged erythrocytes, that is after the major enrichment of neutral sialidase had been accomplished. A second observed difference is the higher susceptibility to PIPLC hydrolysis by the acidic sialidase in the young than old erythrocytes (88.4% against 66.6% of solubilization). This finding is not surprising since a portion of acidic sialidase is presumably not linked to the erythrocyte membrane by a GPI anchor [36,38] or, if so, may contain an additional hydrophobic tail that strengthens its binding to the membrane. It can be assumed that with ageing the budding process leading to release of acidic sialidase involves preferentially the membrane domains containing the PIPLC sensitive form of the enzyme.

Behavior of acidic and neutral sialidases in *in vitro* membrane vesiculation of erythrocytes of different age

The concept that senescent erythrocytes have a plasma membrane composition different from that of young erythrocytes, and that these differences flag old erythrocytes for macrophage recognition is well established [1,5,21,26,27, 32,63]. It is also a consolidated notion that during ageing a continuous process of membrane budding occurs, with release of vesicles with a different composition than that of the starting plasma membranes [7–11,47,61,63]. This process produces remnant plasma membranes (those of senescent erythrocytes) compositionally different from the starting ones. In order to study the behaviour of acidic and neutral sialidases upon erythrocyte vesiculation we employed the study model of

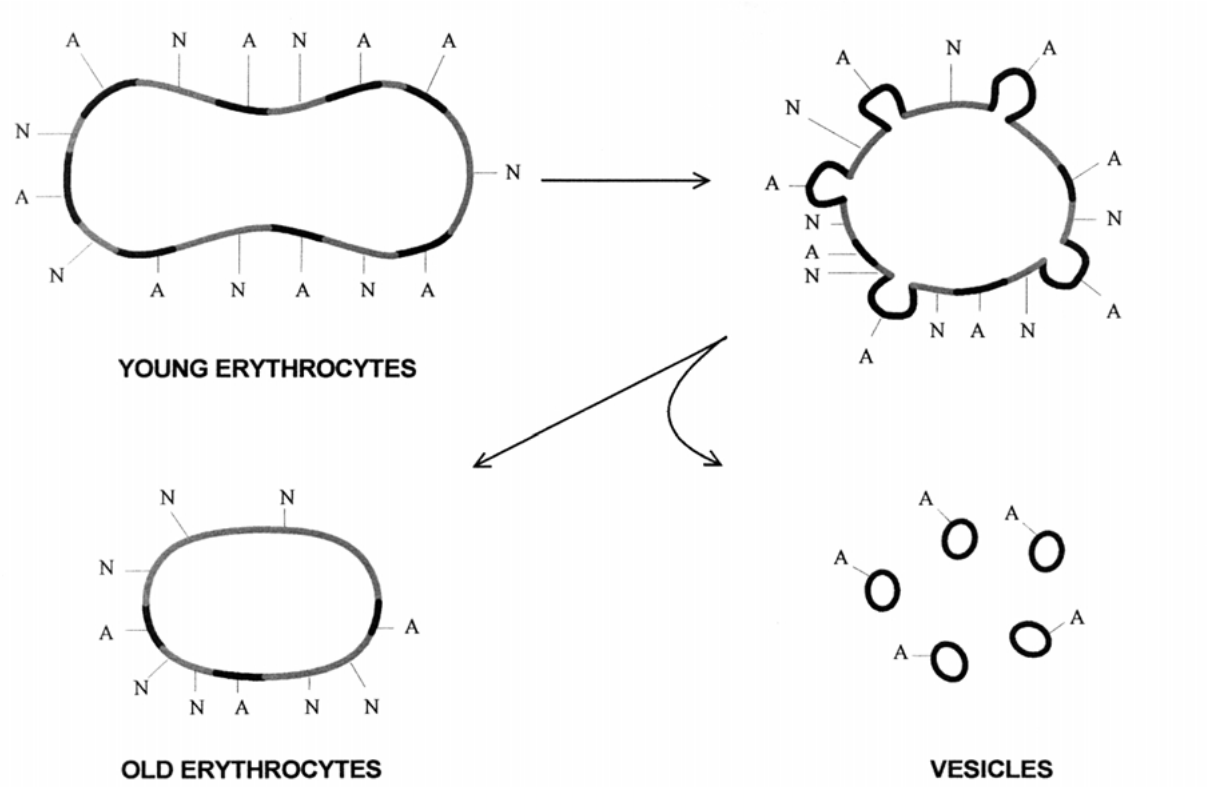

Figure 2. Suggested scheme for the behaviour of ghost linked acid (A) and neutral (N) sialidases in the process of erythrocyte vesiculation elicited by Ca^{2+} loading, possibly mimicking what happens in erythrocyte physiological ageing.

red cells loading with Ca^{2+} by the use of the Ca^{2+}-ionophore A23187, and separation of the formed "vesicles" [47]. We observed that the "vesicles" prepared from either unfractionated and differently aged erythrocytes carried the acidic sialidase but did not contain the neutral sialidase. The latter enzyme remained firmly bound to the remnant erythrocytes and underwent a marked increase in specific activity (referred to phospholipid phosphorus) as compared to the starting erythrocytes. Noteworthy, the sialoglycoconjugates of both lipid and protein nature were almost unaffected by the vesiculation event, their specific concentration (referred to phospholipid phosphorus) in "vesicles" being only 1.4% that of the starting erythrocytes. Therefore, upon vesiculation the plasma membrane of remainder erythrocytes looses acidic sialidase and becomes enriched in neutral sialidase, largely reflecting what happens to erythrocytes with ageing. There is to add that the vesicles produced by the Ca^{2+} loading procedure carry an acidic sialidase almost completely (93.5%) releasable by PIPLC, similarly to two other GPI-anchored proteins, Ach-esterase and Decay Accelerating Factor [63]. These findings support the hypothesis that the vesiculation process takes place at, and involves, specific membrane microdomains (rich also in sphingomyelin—presumably the Triton X-100 resistant microdomains [63,64,65]—which preexist in the erythrocyte membranes. Further favor to this hypothesis comes from the findings that the acidic sialidase is almost completely resistant to Triton X-100 treatment [38], and that erythrocyte vesiculation leads to release of diacylglycerol together with sphingomyelin [7,47].

In conclusion, this work shows that human erythrocytes undergo a marked impoverishment of acidic sialidase with ageing, and a concomitant striking relative enrichment in neutral sialidase, the ratio between these two enzymes being diagnostic of erythrocyte age. A second message is that in the *in vitro* model of erythrocyte vesiculation based on Ca^{2+} loading, the acidic sialidase is released with "vesicles", whereas the neutral sialidase remains firmly bound to the membrane of the remainder erythrocytes (see Figure 2). The marked enrichment with age of the neutral sialidase, the ability of this enzyme to affect sialoglycoproteins, including the endogenous ones, the confirmation (provided here) that the ghost membranes of old erythrocytes have a lower content of sialoglycoconjugates (especially sialoglycoproteins) than young erythrocytes, and the recent evidence [27] that into the glycoconjugates from old erythrocytes more sialic acid can be incorporated than the young cells, give new support to the hypothesis that sialic acid is involved in the control of erythrocyte life span and suggest a role for neutral sialidase in the loss of sialic acid occurring during erythrocyte ageing. Investigations are in progress aimed at ascertaining the possible involvement of sialidases in the molecular events that elicit membrane vesiculation occurring during erythrocyte ageing.

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References

1 Lutz HU, Flepp R, Stingaro-Wipf G, Naturally occurring autoantibodies to exoplasmic and cryptic region of band 3 protein, the major integral membrane protein of human red blood cells, *J Immunol* **133**, 2610–18 (1984).
2 Cooper RA, Jandl JH, Destruction of erythrocytes. In *Haematology,* Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds), New York, McGraw-Hill Book, 1986, pp. 377–88.
3 Kay MMB, Mechanism of removal of senescent cells by human macrophages *in situ*, *Proc. Natl. Acad. Sci USA* **72**, 3521–25 (1975).
4 Magnani M, De Flora A, *Red Blood Cell Ageing,* Magnani M, De Flora A (eds), New York and London, Plenum Press 1991, pp. 307.
5 Lutz HU, *Blood Cell Biochemistry: Erythroid Cells,* Harris JR (ed), New York and London, Plenum Press, 1990, vol.1, pp. 81–120.
6 Kiefer CR, Snyder LM, Oxidation and erythrocyte senescence, *Curr Opin Hematol* **7**, 113–16 (2000).
7 Allan D. Billah MM, Finean JB, Michell RH, Release of diacylglicerol-enriched vesicles from erythrocytes with increased intracellular [Ca^{2+}], *Nature* **261**, 58–60 (1976).
8 Lutz HU, Liu S, Palek J, Release of spectrin-free vesicles from human erythrocytes during ATP depletion *J Cell Biol* **73**, 548–57 (1977).
9 Cookley WT, Bater AJ, Deeley JOT, Vesicles production on heated and stressed erythrocytes *Biochim Biophys Acta* **512**, 318–30 (1978).
10 Whitlow M, lida K, Marshall P, Silber R, Nussenzweig V, Cells lacking phosphatidylinositol-linked proteins have impaired ability to vesiculate *Blood* **81**, 510–16 (1993).
11 Wagner GM, Chiu DTY, Yee MC, Lubin NH, Red cell vesiculation—a common membrane physiological event, *J Lab Clin Med* **108**, 315–24 (1986).
12 Kelm S, Schauer R, The galactose recognising system of rat peritoneal macrophages; identification and characterisation of the receptor molecule, *Hoppe Seyler's Z Physiol Chem* **369**, 693–705 (1988).
13 Kannan R, Yuan J, Low PS, Isolation and partial characterisation of antibody- and globin-enriched complexes from membranes of dense human erythrocytes, *Biochem J* **278**, 57–62 (1991).
14 Ando K, Kikugawa K, Beppu M, Induction of band 3 aggregation in erythrocytes results in anti-band 3 auto antibody binding to the carbohydrate epitopes of band 3, *Arch Biochem Biophys* **339**, 250–57 (1997).
15 Low PS, Waugh SM, The role of haemoglobin denaturation and band 3 clustering in red blood cell ageing, **227**, 531–33 (1985).
16 Schlüter K, Drenckhahn D, Co-clustering of denatured haemoglobin with band 3: its role in binding of auto-antibodies against band 3 to abnormal and aged erythrocytes, *Proc Natl Acad Sci USA* **83**, 6137–141 (1986).
17 Turrini F, Arese P, Yuan J, Low PS, Clustering of integral membrane proteins of human erythrocyte membrane stimulates autologous IgG binding, complement deposition, and phagocytosis, *Blood* **81**, 3146–152 (1991).
18 Beppu M, Mizukami A, Nagoya M, Kikugawa K, Binding of anti-band 3 autoantibody to oxidatively damaged erythrocytes, *J Biol Chem* **265**, 3226–233 (1990).
19 Ando K, Beppu M, Kikugawa K, Nagai R, Hoiuchi S, Membrane proteins of human erythrocytes are modified by advanced glycation end products during aging in the circulation, *Biochem Biophys Res Comm* **258**, 123–27 (1999).
20 Brovelli A, Pallavicini G, Sinigaglia F, Balduini CL, Balduini C, Identification of a sialoglycopeptide released by self-digestion from human erythrocyte membranes, *Biochem J* **158**, 497–500 (1976).
21 Corsi D, Paiardini M, Crinelli R, Bucchini A, Magnani M, Alteration of α-spectrin ubiquitination due to age-dependent changes in the erythrocyte membrane, *Eur J Biochem* **261**, 775–83 (1999).
22 Bocci V, The role of sialic acid in determining the life-span of circulating cells and glycoproteins, *Experientia* **32**, 135–42 (1976).
23 Cohen NS, Ekholn JE, Luthra MG, Hananan DJ, Biochemical characterisation of density-separated human erythrocytes, *Biochim Biophys Acta* **419**, 229–42 (1976).
24 Jancik J, Schauer R, Sialic acid. Determinant of the life-time of rabbit erythrocytes, *Hoppe Seyler's Z Physiol Chem* **355**, 395–406 (1974).
25 Schlepper-Schäfer J, Kolb-Bachofen V, Kolb H, Identification of a receptor for senescent erythrocytes on liver macrophages, *Biochem Biophys Res Comm* **115**, 551–58 (1983).
26 Aminoff D, The role of sialoglycoconjugates in the aging and sequestration of red cells from circulation, *Blood Cells* **14**, 229–35 (1988).
27 Bratosin D, Mazurier J, Debray H, Lecolq M, Boilly B, Alonso C, Moisei M, Motas C, Montreuil J, Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins. Evidence that sialic acid control their life span, *Glycoconjugate J* **12**, 258–67 (1995).
28 Aminoff D, Bell WC, Fulton I, Ingebrightsen N, Effect of sialidase on the viability of erythrocytes in circulation, *Am J Haematol* **1**, 419–28 (1976).
29 Greenwalt TJ, Steane EA, Quantitative hemagglutination: V. Influence of *in vivo* aging and neuraminidase treatment on the M and N antigens of human red cells, *Br J Haematol* **25**, 217–21 (1973).
30 Schauer R, Chemistry, metabolism and biological functions of sialic acid, *Adv Carbohydr Chem Biochem* **40**, 131–234 (1982).
31 Ando K, Kikugawa K, Beppu M, Involvement of sialylated poly-N-acetyllactosaminyl sugar chains of band 3 glycoprotein on senescent erythrocytes in anti-band 3 autoantibody binding, *J Biol Chem* **269**, 19394–398 (1994).
32 Shinozuka T, Miyata Y, Takei S, Yoshida R, Ogamo A, Nakagawa Y, Kuroda N, Yanagida J, Changes in En(a-)human red blood cell membranes during *in vivo* ageing, *Mech Ageing Develop* **86**, 27–37 (1996).
33 Gielen W, Etzrodt J, Uhlenbruck G, Uber eine neuraminidase in den thrombozyten des rindes, *Thromb Diath Haemorrh* **22**, 203–6 (1969).

34 Bosmann HB, Red cell hydrolases III. Neuraminidase activity in isolated human erythrocyte plasma membranes, *Vox Sanguinis* **26**, 497–509 (1974).
35 Sagawa J, Miyagi T, Tsuiki S, Characterisation of the major sialidases of various types of rat blood cell: their comparison with rat liver sialidases, *J Biochem* (Tokyo) **107**, 452–56 (1990).
36 Chiarini A, Fiorilli A, Di Francesco L, Venerando B, Tettamanti G, Human erythrocyte sialidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor and partly located on the outer surface, *Glycoconjugate J* **10**, 64–71 (1993).
37 Chen XG, Nagai T, Yamada H, Sialidase in rabbit blood. Characterisation of sialidase purified from rabbit erythrocyte membrane, *Eur J Biochem* **221**, 655–60 (1994).
38 Venerando B, Fiorilli A, Croci GL, Tettamanti G, Presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH, *Blood* **90**, 2047–56 (1997).
39 Svennerholm L, Quantitative estimation of sialic acid. II – A colorimetric resorcinol-hydrochloric acid method, *Biochim Biophys Acta* **24**, 604–7 (1957).
40 Beutler E, Blume KG, Kaplan JC, Lhor JW, Ramot B, Valentine WN, International Committee for standardisation in haematology: recommended methods for red-cell enzyme analysis, *Br J Haematol* **35**, 331–35 (1977).
41 Salvo G, Caprari P, Samoggia P, Mariani G, Salvati AM, Human erythrocyte separation according to age on a discontinuous Percoll density gradient, *Clin Chim Acta* **122**, 293–305 (1982).
42 Mosca A, Paleari R, Modenese A, Rossini S, Parma R, Rocco C, Russo V, Caramenti G, Paderi ML, Galanello R, Clinical utility of fractionating erythrocytes into Percoll density gradients. In *Red Blood Cell Aging,* Magnani M, De Flora A (eds), New York, Plenum Press, 1991, pp. 227–238.
43 Fehr J, Knob M, Comparison of red cell creatine level and reticulocyte count in appraising the severity of haemolytic processes, *Blood* **53**, 966–76 (1979).
44 Griffiths WJ, The determination of creatine in body fluids and muscle, and phosphocreatine in muscle, using the autoanalyzer, *Clin Chim Acta* **9**, 210–14 (1964).
45 Steck TL, Kant JA, Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes, *Methods Enzymol* **31**, 172–85 (1974).
46 Steck TL, Weinstein RS, Straus JH, Wallach DF, Inside-out red cell membrane vesicles: preparation and purification, *Science* **168**, 255–56 (1970).
47 Allan D, Thomas P, Limbrick AR, The isolation and characterisation of 60 nm vesicles (nanovesicles) produced during ionophore A23187-induced budding of human erythrocytes, *Biochem J* **188**, 881–87 (1980).
48 Iida K, Whitlow MB, Nussenzweig V, Membrane vesiculation protects erythrocytes from destruction by complement, *J Immunol* **199**, 2638–42 (1991).
49 Caimi L, Lombardo A, Preti A, Wiesmann U, Tettamanti G, Optimal conditions for the assay of fibroblast neuraminidase with different natural substrates, *Biochim Biophys Acta* **571**, 137–45 (1979).
50 Tettamanti G, Bonali F, Marchesini S, Zambotti V, A new procedure for the extraction, purification and fractionation of brain gangliosides, *Biochim Biophys Acta* **296**, 160–68 (1973).
51 Lowry OM, Rosebrough NJ, Farr AL, Randall RJ, Protein measurement with Folin-phenol reagent, *J Biol Chem* **193**, 265–69 (1951).
52 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding, *Anal Biochem* **72**, 248–50 (1976).
53 Ellman GL, Courtney DK, Andreas V, Featherstone RM, A new and rapid colorimetric determination of acetyl cholinesterase activity, *Biochem Pharmacol* **7**, 88–91 (1961).
54 Ladish S, Gillard B, Isolation and purification of phospholipids and gangliosides from plasma, *Methods Enzymol* **138**, 300–6 (1987).
55 Bartlett GR, Phosphorus assay in column chromatography, *J Biol Chem* **234**, 466–72 (1959).
56 Taguchi R, Mizuno M, Inoue M, Increase in osmotic fragility of bovine erythrocytes induced by bacterial phospholipase C, *J Biochem* (Tokyo) **93**, 403–8 (1983).
57 Snedecor GW, Cochran WC, *Statistical Methods,* Iowa State University Press Ann IA (1967).
58 Rennie CM, Thompson S, Parker AC, Maddy AH, Human erythrocyte fraction in "Percoll" density gradients, *Clin Chim Acta* **98**, 119–208 (1979).
59 Gutowski KA, Hudson JL, Aminoff D, Flow cytometric analysis of human erythrocytes: I. Probed with lectins and immunoglobulins, *Exp Gerontol* **26**, 315–26 (1991).
60 Winterbourn CC, Batt RD, Lipid composition of human red cells of different ages, *Biochim Biophys Acta* **202**, 1–8 (1970).
61 Lutz HU, Vesicles isolated from ATP-depleted erythrocytes and out of thrombocyte-rich plasma, *J Supramol Struct* **8**, 375–82 (1978).
62 Rolfes-Curl A, Ogden CL, Omann GM, Aminoff D, Flow cytometric analysis of human erythrocytes. II Possible identification of senescent RBC with flourescently labeled wheat germ agglutinin, *Exp Gerontol* **26**, 327–45 (1991).
63 Bütikofer P, Kuypers FA, Xu CM, Chiu DTY, Lubin B, Enrichment of two glycosyl-phosphatidylinositol-anchored proteins, acetylcholinesterase and decay accelerating factor, in vesicles resealed from human red blood cells, *Blood* **74**, 1481–85 (1989).
64 Hooper NM, Turner AJ, Ectoenzymes of kidney microvillar membrane. Differential solubilization by detergents can predict a glycosyl-phosphatidylinositol membrane anchor, *Biochem J* **250**, 865–72 (1988).
65 Simons K, Ikonen E, Functional rafts in cell membranes, *Nature* **387**, 569–72 (1997).

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