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Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins. Evidence that sialic acids control their life span*

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Comparing the properties of 'young' and senescent ('aged') O^+ erythrocytes isolated by applying ultracentrifugation in a self-forming Percoll gradient, we demonstrate that the sialic acids of membrane glycoconjugates control the life span of erythrocytes and that the desialylation of glycans is responsible for the clearance of the aged erythrocytes. This capture is mediated by a β-galactolectin present in the membrane of macrophages. The evidence supporting these conclusions is as follows:

(1) Analysis by flow cytofluorimetry of the binding of fluorescein isothiocyanate labelled lectins specific for sialic acids shows that the aged erythrocytes bind less WGA, LPA, SNA and MAA than young erythrocytes. The binding of DSA and LCA is not modified. On the contrary, the number of binding sites of UEA-I specific for O antigen and of AAA decreases significantly. PNA and GNA do not bind to erythrocytes.

(2) RCA_{120} as well as *Erythrina cristagalli* and *Erythrina corallodendron* lectins specific for terminal β-galactose residues lead to unexpected and unexplained results with a decrease in the number of lectin binding sites associated with increasing desialylation.

(3) The glycoconjugates from the old erythrocytes incorporate more sialic acid than the young cells. This observation results from the determination of the rate of transfer by α–2,6-sialyltransferase of fluorescent or radioactive *N*-acetylneuraminic acid, using as donors CMP-9-fluoresceinyl-NeuAc and CMP-[^{14}C]-NeuAc, respectively.

(4) Microscopy shows that the old erythrocytes are captured preferentially by the macrophages relative to the young ones. Fixation of erythrocytes by the macrophage membrane is inhibited by lactose, thus demonstrating the involvement of a terminal β-galactose specific macrophage lectin.

(5) Comparative study of the binding of WGA, LPA, SNA and MAA to the aged erythrocytes and to the *in vitro* enzymatically desialylated erythrocytes shows that the desialylation rate of aged cells is low but sufficient to lead to their capture by the macrophages.

Keywords: Senescent erythrocytes, lectins, flow cytofluorimetry, sialic acid, erythrophagocytosis, macrophages, endogeneous galactolectin.

Abbreviations: BSA, bovine serum albumin; CMP-NeuAc, cytidine monophosphate *N*-acetylneuraminate; CSB, cell sialylation buffer; EDTA, ethylene diamine tetraacetic acid; FITC, fluoresceinyl isothiocyanate; 9-FITC-NeuAc, 9-fluoresceinyl-*N*-acetylneuraminate; NeuAc, *N*-acetylneuraminic acid; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate buffer saline solution; PMSF, phenylmethyl-sulfonyl fluoride; RBC, red blood cells; SCA, Senescent Cell Antigen; SDS, sodium dodecyl sulfate; SFG, senescent factor glycopeptides.

Lectins: AAA, *Aleuria aurantia* agglutinin; DSA, *Datura stramonium* agglutinin; ECA, *Erythrina cristagalli* agglutinin; GNA, *Galanthus nivalis agglutinin*; LCA, *Lens culinaris* agglutinin; LFA, *Limax flavus* agglutinin; LPA, *Limulus polyphemus* agglutinin; MAA, *Maackia amurensis* agglutinin; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; SNA, *Sambucus nigra* agglutinin; UEA-I, *Ulex europeus* agglutinin-I; WGA, Wheat germ agglutinin.

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Introduction

Human erythrocytes have a definite life span of 120 days in the circulation after which they are captured and endocytosed by macrophages. This evidence raises the following questions which have been asked for a long time: (i) what signals the death sentence of red blood cells and (ii) what are the physiological mechanisms of sequestration from the blood stream with such precision. In other words, the question is: 'By what signal do the macrophages distinguish between young and old erythrocytes?'. This problem continues to give rise to controversy, and several hypotheses have been proposed which await confirmation (for reviews see [1–11]). One of these has been proposed by Kay (for recent reviews see [11]) and is based on the concept of the senescent cell antigen (SCA) an ageing antigen postulated in 1975 [12, 13]. This antigen appears in senescent cells, and binds to naturally occurring IgG autoantibodies so initiating the removal of the cells by macrophages mediated through Fc-gamma or C3b receptors. In the case of erythrocytes, the ageing antigen would derive from the cleavage of the protein moiety of the so-called band 3 [14–17]. Hypotheses have also been proposed describing the demasking of cryptic signals: (i) by action of endopeptidases on membrane glycoproteins [18, 19], a hypothesis recently reinforced by the characterization in RBC membrane of a novel high molecular mass peptidase sensitive to PMSF [20]; or (ii) by remodelling of the erythrocyte membrane by the loss of vesicles *in vivo* [21–23] or *in vitro* [24, 25]. In order to explain the formation of these vesicles, it has been suggested by Bocci [18] that segments of membranes might be pinched off as erythrocytes are squeezed through small vascular apertures. This results in an overall loss of surface membrane carbohydrate components such that all the constituent sugars are lost in the same proportions.

In another hypothesis, the sialic acids of membrane glycoconjugates play a key role in the life span of erythrocytes. This hypothesis is founded on results obtained as far back as 1955 by Stewart *et al.* [26] who demonstrated that neuraminidase treated red blood cells were sequestered more quickly by reticuloendothelial macrophages than the native cells (for review see [1, 5]). This original observation was later confirmed by Halbhuber *et al.* [27], Schauer *et al.* [28–30], Kolb *et al.* [31] and Aminoff *et al.* [32–37]. These authors demonstrated that neuraminidase treatment of erythrocytes, by demasking the penultimate β-galactose residues of erythrocyte membrane glycoconjugate glycans, induces the capture of the desialylated cell mediated by a β-galactose specific lectin present in the macrophage membrane and later isolated by Schauer *et al.* [38]. This capture results *in vivo* in a rapid sequestration of neuraminidase-treated erythrocytes from the blood stream. This hypothesis first founded on *in vitro* enzymatic desialylation of erythrocytes has been extended to a physiological *in vivo* desialylation during ageing of RBCs [29–37, 39].

On the basis of Schauer's concept we have undertaken a comparative study of the sialylation rate of young and aged erythrocytes separated in a self-forming Percoll gradient according to Lutz *et al.* [40]. The following methods have been used: (i) determination of fluorescent specific lectin binding by flow cytofluorimetry; (ii) measurement of the enzymatic transfer of N-acetylneuraminic acid onto erythrocyte membrane; (iii) microscopic visualization of the capture of erythrocytes by macrophages.

The present work contributes to the demonstration that the physiological desialylation of erythrocytes is the main factor in their capture by macrophages.

Materials and methods

Materials

Human blood type ORh$^+$ collected in heparin was kindly furnished by the Centre Régional de Transfusion Sanguine de Lille. Drabkin's reagent for determination of haemoglobin (Hemotrol) was from Bio-Mérieux (Marcy-L'Etoile, France). Percoll solution (density: 1.130 ± 0.005 gml^{-1}) was obtained from Pharmacia-Biotech (St Quentin-Yvelines, France). PMSF and fluoresceinyl-isothiocyanate lectins (FITC-lectins) were from Sigma (St Louis, MO, USA) for *Erythrina cristagalli* agglutinin and *Erythrina corallodendron* agglutinin, GNA, LCA, LPA, MAA, PNA, RCA_{120}, UEA-I and WGA. DSA and SNA were from EY Laboratories (San Mateo, CA, USA). FITC-AAA was prepared in our laboratory. Cytidine-5′-monophospho-9-(3-fluoresceinyl-thioureido)-9-deoxy-*N*-acetyl-neuraminic acid (CMP-9-fluoresceinyl NeuAc) and CMP-*N*-acetylneuraminate:β-D-galactosyl-1,4-*N*-acetyl-β-D-glucosamine, α-2,6-*N*-acetyl neuraminyl-transferase E.C. 2.4.99.1 (α-2, 6-sialyltransferase) were from Boehringer (Mannheim, Germany). Cytidine-monophosphate-[^{14}C]-*N*-acetyl-neuraminic acid (CMP-[^{14}C]-NeuAc) (specific radioactivity: 256 μCi mM^{-1}) was furnished by Amersham (Amersham, UK). *Vibrio cholerae* neuraminidase E.C. 3.2.1.18 was from Calbiochem (La Jolla, USA). The cytofluorimeter used was a FACScan apparatus from Becton-Dickinson (San Jose, CA, USA) and the coulter counter model ZF from Analis (Namur, Belgium).

Isolation of red blood cells from whole blood

Heparinized human blood was processed within 1 h of collection. Cells were sedimented by centrifugation (2000 × g; 4°C; 5 min). After removal of plasma platelets and leukocytes by aspiration, cells were washed three-times with PBS buffer containing 0.2 mM PMSF as antiproteases.

Separation of young and old erythrocytes

Young and old erythrocytes were separated by ultracentrifugation in a self-forming Percoll gradient according to the procedure of Lutz *et al.* [40]. Briefly, pelleted cells were resuspended to give a haematocrit of 15% in a Percoll buffer, pH 7.4, osmolality 320–330 mosmol kg^{-1} (427 g Percoll, 114 mM NaCl, 0.5% glucose, 10 mM phosphate, 0.5M EDTA, 30

μg ml^{-1} PMSF in a final volume of 500 ml). The suspension was centrifuged in 40 ml tubes at 33 000 × g at 4°C for 1 h. Cells immediately surrounding the main fraction of erythrocytes were collected by aspiration and constitute the young erythrocyte population. After elimination of the principal intermediate band by aspiration, the remaining lower fraction was collected and represents the old erythrocyte population. Both fractions were washed three-times with PBS buffer containing 0.2 mM PMSF and recentrifuged in Percoll gradient as described above. Freedom from non-erythrocyte contaminants was ascertained by the determination of the haemoglobin content per cell and by the dot-plot flow cytometry of the whole population of RBCs (designated as 'total erythrocytes') taken as reference. Percentage of young and old erythrocytes with regard to the total RBC population was determined by using a coulter counter.

In vitro *desialylation of erythrocytes*

Vibrio cholerae neuraminidase solution corresponding to 0.15 U of enzyme (10 μl) was added to 1 ml of erythrocyte suspension (haematocrit of 30%) in PBS buffer pH 7.4. After incubation (37°C; 1 h) under constant stirring, cells were centrifuged (2000 × g; 4°C; 5 min) and washed three-times with PBS buffer. They were further used as reference standards for all of the subsequent experiments of lectin binding, *N*-acetylneuraminic acid transfer and capture by macrophages described below.

Flow cytometry analysis

Data were collected on a Becton Dickinson FACScan cytofluorimeter. The light-scatter channels were set on linear gains and the fluorescence channels on a logarithmic scale. Cells in suspension in isotonic PBS buffer pH 7.4, osmolality 320–330 mosmol kg^{-1}, were gated for forward and side-angle scatters and 5000 fluorescent particles of each gated population were analysed.

Analysis of the binding of fluorescently labelled lectins to erythrocytes

A solution (50 μl) of FITC-lectins in PBS-PMSF buffer was added to 50 μl of RBC suspension corresponding to 2 × 10^6 total, young or old erythrocytes distributed in 96 well microtitre plates. After 1 h incubation at 4°C in the dark, cells were washed three-times with PBS-PMSF buffer and centrifuged (2000 × g; 4°C; 5 min).

The binding for each lectin was first studied at concentrations ranging from 0 to 50 mM in order to determine the optimal lectin concentration avoiding haemagglutination. In addition, the non-specific binding was determined in the presence of specific inhibitors at a concentration of 0.1 M. Results were expressed as equivalent fluorescent bound particles calculated from the mean value of the logarithm of fluorescence intensity. Binding capacity and dissociation constants were calculated according to Scatchard [41] using the Enzfitter Biosoft software (Cambridge, UK).

This experimental protocol was applied to the following FITC-labelled lectins: LPA, MAA, SNA, WGA, PNA, RCA_{120}, *Erythrina cristagalli* agglutinin, *Erythrina corallodendron* agglutinin, GNA, DSA, AAA, LCA and UEA-I.

Determination of N*-acetylneuraminic acid transfer onto erythrocytes*

The relative amounts of β-galactose terminal residues present on the RBC surface was determined by measuring the rate of *N*-acetylneuraminic acid transfer onto erythrocytes using an α-2,6-sialyltransferase (the only commercial sialyltransferase presently available) and two CMP-NeuAc donors: CMP-[^{14}C]NeuAc [42] and CMP-9-amino-FITC-NeuAc [43]. In both cases the optimal concentration of enzyme was kinetically determined.

Transfer of [^{14}C]NeuAc CMP-[^{14}C] NeuAc corresponding to 0.25 μCi and 2 mU of α-2,6-sialyltransferase dissolved in CSB buffer, pH 7.4, were added to a suspension of 8 × 10^7 erythrocytes in 100 μl of CSB buffer (PBS buffer, pH 7.4, containing 5 mg ml^{-1} BSA and 1 mg ml^{-1} glucose). After incubation (2 h; 37°C) under constant stirring, the cells were washed three-times with PBS buffer. After centrifugation (2000 × g; 4°C; 10 min) the erythrocytes were lysed using 100 μl of a 1% SDS solution in PBS and the membrane bound radioactivity was determined.

Transfer of 9-amino-fluoresceinyl-NeuAc To 2 × 10^7 erythrocytes in suspension in 100 μl of CSB buffer, pH 7.4, were added 0.5 μg of CMP-9-amino-fluoresceinyl-NeuAc dissolved in 5 μl of CSB and 0.2 mU of α-2,6-sialyltransferase dissolved in CSB buffer. After incubation (37°C in the dark) for 2 h under constant stirring, RBCs were washed and centrifuged as described above. The rate of transfer was determined by measuring the mean fluorescence intensity of erythrocytes and by determining the percentage of erythrocytes which had been resialylated.

Capture of erythrocytes by macrophages

The capture of normal, young, old and neuraminidase treated erythrocytes by macrophages was carried out according to Kelm and Schauer [38] and observed by microscopy with an inverted phase contrast microscope Olympus IMT2.

Mouse spleen macrophages, 2 to 4 × 10^4, in suspension in 200 μl of culture medium 199 E containing 30% of homologous serum were incubated (37°C; 1 h; humidified air containing 5% CO_2) in plastic micro-vials. Non-adherent macrophages were eliminated by two washings with cold 199 E medium. The adherent macrophages were covered with 200 μl of cold 199 E medium. After 30 min at 4°C, 20 μl of human erythrocyte suspension in 199 E medium corresponding to 10^7 cells, and 100 μl of murine serum were added. After 1 h incubation at 37°C, cells were washed three-times with cold 199 E medium and observed with a phase-contrast microscope. The same experiments were carried out in presence of 16 μl of a 1 M lactose solution.

Results

Fractionation of erythrocytes from whole blood into young and senescent erythrocyte populations

The method of Lutz *et al.* [40] based on the demonstration by Linderkamp and Meiselman [44] that senescent erythrocytes possess a higher density and a smaller size than the young ones leads to the rapid isolation in a good yield of both erythrocyte populations. The homogeneity of each population was verified: (i) by recentrifugation in Percoll gradient which furnished single and well delimited bands (not shown); and (ii) by the dot-plot patterns obtained by flow cytometry taking into account two criteria – density (side scatter scale) and size (forward scatter scale) of the cells studied. As shown in Figs 1 and 2, the whole erythrocyte population is very heterogeneous (Figs. 1T and 2T). However, it is possible to localize the young and old erythrocytes area. In addition, Figs 1O and Y as well as Figs 2O and Y show that Lutz's method furnishes very enriched populations of young and old erythrocytes with few contaminant cells. The percentage of cells isolated as young and old erythrocytes was estimated at 1.0 ± 0.2% for both populations. These values are similar to those obtained by Sorette *et al.* [45]. Both populations were found to contain the same amount of haemoglobin confirming the results obtained by previous authors.

Flow cytofluorimetric analysis of binding of FITC-lectins

The kinetics of binding was carried out for each FITC-lectin as described in Materials and methods in order to avoid haemagglutination. In addition, the non-specific binding using specific inhibitors was determined. In the following paragraph, we describe in detail experiments and results obtained only with WGA since the results given by each of the other lectins were similar.

Binding of FITC-WGA to erythrocytes

The binding of FITC-WGA to erythrocytes was concentration dependent with saturation occurring at a lectin concentration of 0.8 nM for total and old erythrocytes and of 1.8 nM for young erythrocytes as shown in Fig. 3. The binding was inhibited by *N*-acetylglucosamine and *N*-acetylneuraminic acid at a concentration of 0.1 M (Fig. 4) thus demonstrating the reversibility and the specificity of the interaction. Furthermore, the inhibition by *N*-acetylneuraminic acid was greater than that with *N*-acetylglucosamine indicating that WGA has a higher affinity for *N*-acetylneuraminic acid than for *N*-acetylglucosamine.

Scatchard analysis of the results shown in Fig. 3 and in Table 1 revealed that the apparent equilibrium constant K_d was approximately 3 and 1.9 nM for total and old erythrocytes respectively, and that the number of binding sites could be estimated to be 40 000 and 37 000, respectively. Results calculated in the same way showed that the binding to young erythrocytes was significantly different: K_d : 5.80 nM; number of sites: 60 000, thus demonstrating that the number of sialic acid residues of young erythrocytes is higher than that of old erythrocytes.

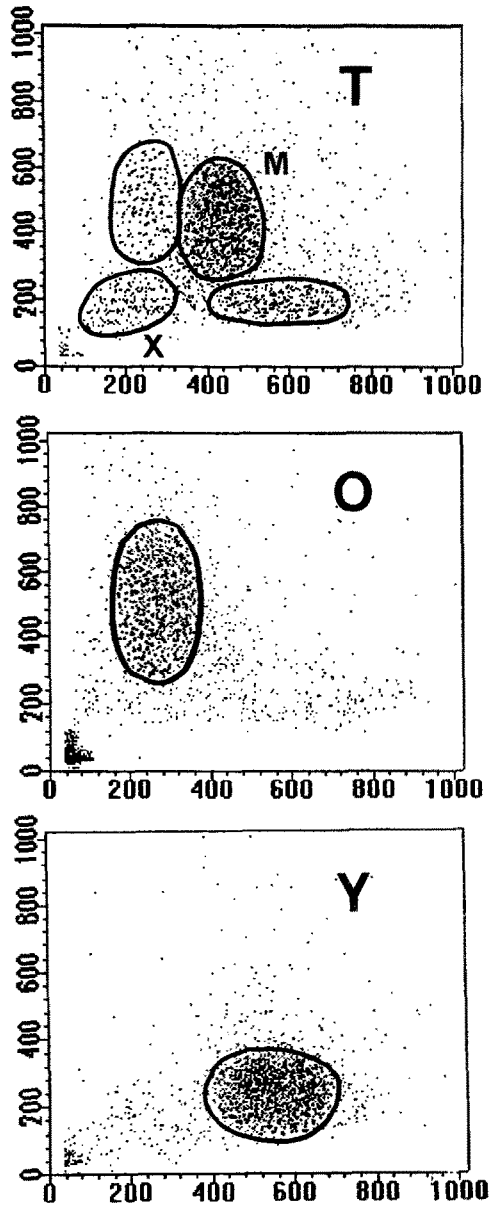

Figure 1. Dot-plot analysis of total (T), old (O) and young (Y) erythrocyte populations. Abcissae: forward scatter (fluorescence intensity); ordinates: side scatter (fluorescence intensity). In T histogram: M and X represent the mature RBCs and a non-defined RBC population, respectively.

Furthermore, comparison of the number of WGA binding sites of neuraminidase-treated erythrocytes and of old erythrocytes (9000 and 37 000 respectively) leads to the conclusion that the amount of desialylation of senescent RBC does not need to be very great to ensure the capture by macrophages.

Binding studies with other FITC-lectins

The methodology described in detail for FITC-WGA was further applied to the following FITC-lectins: LPA, MAA, SNA, RCA_{120}, *Erythrina cristagalli* agglutinin, *Erythrina*

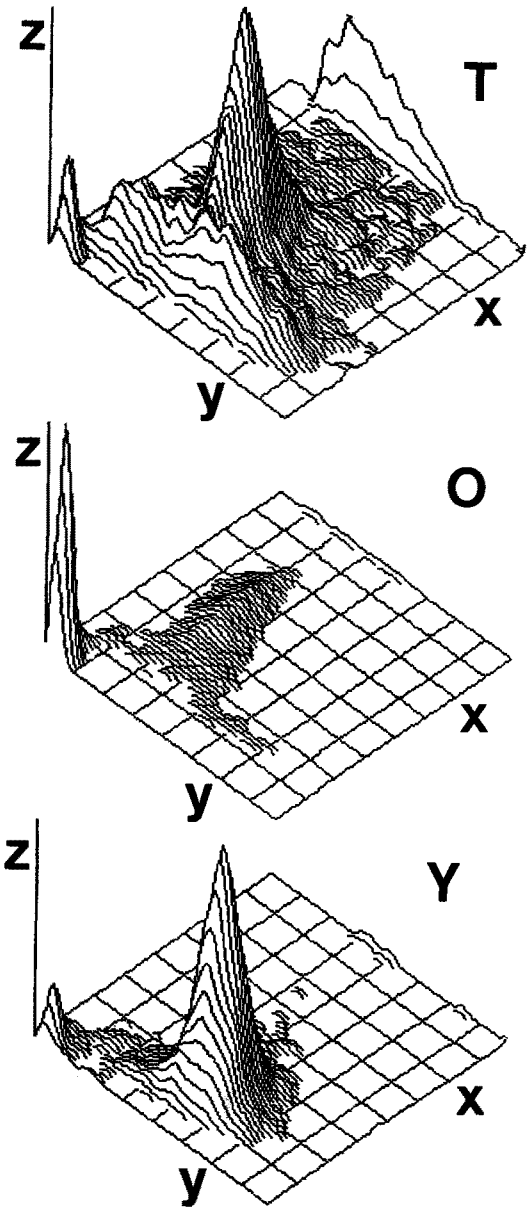

Figure 2. Three dimensional representation of the dot-plot analysis of total (T), old (O) and young (Y) erythrocyte populations. x: side scatter (fluorescence intensity); y: forward scatter (fluorescence intensity); z: cell number

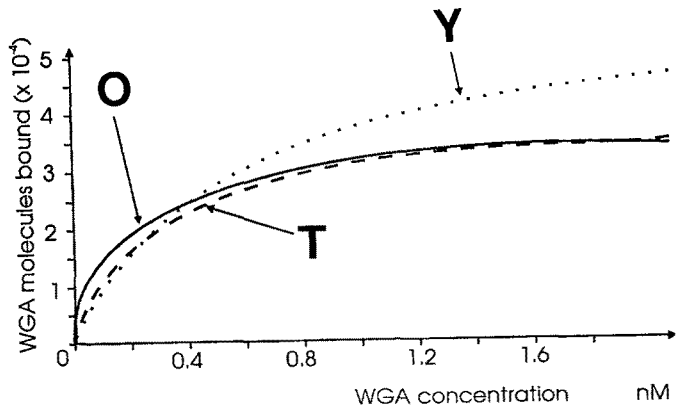

Figure 3. Scatchard analysis of the binding of FITC-WGA to total (T), old (O) and young (Y) erythrocyte populations.

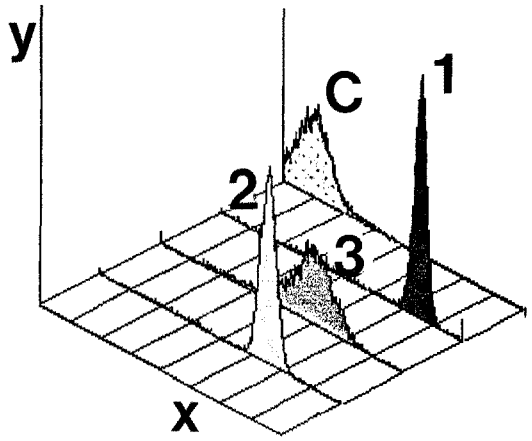

Figure 4. Three dimensional representation of the inhibition of binding of FITC-WGA to erythrocytes. (1): total binding in absence of inhibitor; (2) and (3): binding in presence of inhibitors: *N*-acetylglucosamine and *N*-acetylneuraminic acid, respectively. C: autofluorescence of erythrocytes; x: logarithm of fluorescence intensity; y: cell number.

corallodendron agglutinin, DSA, PNA, LCA, AAA, UEA-I and GNA. The results we obtained led to the following observations.

(i) The results are very reproducible since the standard deviation ranged between ±6–8%. They are reported in Table 1 which gives the results obtained with the erythrocytes of a single donor, except for UEA-I for which the given values are from five different donors.

(ii) The results obtained with LPA, MAA and SNA are similar to those obtained with WGA, and confirm the desialylation of erythrocytes during the ageing process. This loss of sialic acid is slight when compared with desialylation using neuraminidase. However, it was possible to distinguish two categories of lectins, based on the determination of their dissociation constants. In the case of WGA and MAA the desialylation involves ligands with low affinity, as the dissociation constants for the senescent erythrocytes are two to three times weaker. In contrast, the dissociation constants for LPA and SNA show little variation. In addition, comparison of the number of binding sites for α-2,3-sialyl residues detected with MAA and with α-2,6-sialyl residues detected with SNA shows that the loss of binding sites with MAA is greater than for SNA. This could imply that the process of desialylation is essentially concerned with α-2,3-linked sialic acid.

(iii) Contrary to expectations, RCA binding to erythrocytes decreased with progressive desialylation. The same

Table 1. Characteristics of FITC-lectin binding to the membrane of total, young, old and neuraminidase-treated erythrocyte populations determined by flow cytofluorimetry.

		Erythrocytes			
		Total	*Young*	*Old*	*Neuraminidase-treated*
WGA	n	40 000	60 000	37 000	9000
	K_d	2.95	5.80	1.92	–
LPA	n	8800	10 200	8500	1500
	K_d	5.60	4.20	5.00	–
MAA	n	9500	11 000	6300	4500
	K_d	3.24	4.18	1.84	–
SNA	n	11 000	11 000	9200	7000
	K_d	1.97	2.00	2.56	–
RCA_{120}	n	16 000	24 000	16 000	11 000
	K_d	13.00	16.00	13.00	–
Erythrina crystagalli	n	3900	3300	2200	1800
	K_d	3.25	2.70	0.70	–
Erythrina corallodendron	n	3000	2600	2100	1900
	K_d	1.32	0.95	0.70	–
AAA	n	22 700	29 400	20 400	20 000
	K_d	5.59	7.64	5.59	–
LCA	n	3700	3500	3100	1900
	K_d	10.10	14.00	13.00	–
UEA-I	n	36.000	40.000	33.000	ND
	K_d	1.82	1.90	1.95	–
DSA	n	5700	6000	5400	4400
	K_d	2.90	3.20	4.60	–
PNA	No binding				
GNA	No binding				

n: number of binding sites per cell; K_d: dissociation constants (in nM).

result was found with *Erythrina cristagalli* and *Erythrina corallodendron*, which are also specific for terminal β-galactose residues.

(iv) A further unexpected result involves the lack of variation of PNA binding sites. This lectin is specific for Gal(β1-3)GalNAc units which are abundant as mono- and disialylated forms in erythrocyte membrane glycoproteins, in glycophorins in particular. However, this result may be explained by the presence of autoantibodies directed against this structure, the T-antigen, present in human blood (see Discussion).

(v) No significant difference was detected with DSA, specific for poly-*N*-acetyllactosaminic structures, or with the fucolectin LCA. On the contrary, in ageing erythrocytes the number of binding sites of UEA-I and AAA fucolectins which bind to the O antigen decreases by 30% relatively to young erythrocytes for both lectins. However, this occurs without modification of the K_d value in the case of UEA-I.

(vi) GNA, specific for terminal α-mannose residues, does not bind to erythrocytes indicating that there are no oligomannosidic type glycans in erythrocyte membrane glycoproteins.

Enzymatic transfer of N-acetylneuraminic acid onto erythrocyte membranes

Transfer of [^{14}C]N-acetylneuraminic acid

As shown in Table 2, the results we obtained were highly reproducible since the deviation did not exceed ±12% and demonstrate that old erythrocytes accept 2.5-fold more sialic acid than young erythrocytes. This result confirms those obtained by other authors [5, 46] and shows unambiguously that old RBC membrane glycoconjugates contain more sialylable β-galactose terminal residues.

Transfer of 9-amino-fluoresceinyl-N-acetylneuraminic acid

Analysis of histograms shows that all of the erythrocytes (young, old or neuraminidase-treated) are resialylated but at various levels. The old and the neuraminidase-treated erythrocytes constitute a single, homogeneous and persialylated population, the neuraminidase-treated erythrocytes binding more sialic acid residues than the old ones. In fact, the number of transferred sialic acid residues expressed in fluorescein equivalents is 5000–6000 and 19 000–21 000 for old and neuraminidase treated erythrocytes, respectively. In contrast, the resialylation of young erythrocytes is lower and this population is very heterogeneous suggesting that it is resialylated at different levels (up to 4000).

Microscopic observation of erythrocytes capture by macrophages

The phase-contrast microscopy of the incubations of mouse spleen macrophages and the different erythrocyte populations

Table 2. Relative rates[a] of radioactive *N*-acetylneuraminic acid transfer by α-2,6-sialyltransferase to total, young, old and neuraminidase-treated, erythrocyte populations.

	Erythrocytes			
	Total	*Young*	*Old*	*Neuraminidase-treated*
pM[b]	5.8	5.0	12.5	15.6

[a] Deviation: ±12%; [b] pM transferred under the experimental conditions described in Materials and methods.

studied led to the following observations (not shown): (i) very few *young* erythrocytes (varying between 0 and 3) were captured by each macrophage without formation of rosettes; (ii) in the case of *total* RBCs the number of macrophages associated with a higher number of erythrocytes increased and rosettes were found and; (iii) on the contrary, all of the macrophages were covered with *old* erythrocytes and the same aggregates were observed with neuraminidase-treated RBCs. In all cases, the binding of erythrocytes was totally inhibited in the presence of lactose thus confirming that the interactions of erythrocytes and macrophages depend on the presence of a β-galactose-specific lectin present in the macrophage membrane.

Discussion

The flow cytometric-analysis of fluorescent erythrocytes was introduced in 1985 by Green *et al.* [47] for detection of the presence of specific immunoglobulins IgG on the membranes of erythrocytes from patients with sickle cell disease. This involved direct binding of FITC-antihuman IgG to density-separated RBC fractions followed by analyses of the fluorescent cell populations. The use of flow cytometry to follow the ageing of erythrocytes in circulation with fluorescently labelled lectins at the cellular level was introduced with success by Aminoff *et al.* [48, 49]. In fact, since lectins can bind to RBCs at low concentrations without causing agglutination, the flow cytofluorimetry could be applied to detect changes in structural properties between young and senescent erythrocytes taking advantage of differences in their size and shape which are the principal components of the forward lightscatter. Using FITC-labelled WGA, PNA and LFA the authors demonstrated that the smallest erythrocytes isolated by counter-flow centrifugation, corresponding to the oldest ones, showed lower reactivity with FITC-WGA and LFA than the rest of the RBC population. In addition, the reactivity towards these lectins was markedly decreased in the presence of competitive inhibitors of sialic acid as well as after enzymatic removal of this sugar from RBCs. These observations were in agreement with the hypothesis that physiological desialylation is responsible for the clearance of senescent erythrocytes from the circulation. More recently, Sharon and Fibach developed a flow cytometric technique for quantitative measurement of the expression of ABH antigens on RBCs and demonstrated a decrease of A and/or B and H antigens during *in vivo* ageing [50, 51]. As far as we know, the literature concerning the study of the modifications of erythrocyte membrane glycoconjugates during ageing by flow cytofluorimetry probed with fluorescently labelled lectins is limited to the above mentioned articles.

Using the same methodology, we have undertaken a systematic study of the reactivity of isolated young and old erythrocytes towards a series of thirteen FITC-lectins for which the specificity was verified using competitive inhibitors. These were lectins specific for sialic acid residues: WGA, LPA, MAA (α-2,3-sialyl linkages), SNA (α-2,6-sialyl linkages); terminal β-galactosyl residues: RCA_{120}, *Erythrina cristagalli* agglutinin, *Erythrina corallodendron* agglutinin; fucose residues: AAA (α-1,2-, α-1,3- and α-1,6-linkages), LCA (α-1,6-fucosyl + α-mannosyl residues), UEA-I (α-1,2-fucosyl galactose, i.e. O antigen); Gal (β1-3) GalNAc structure: PNA; poly-*N*-acetyllactosaminic sequences: DSA; and terminal α-mannose residues: GNA. For each kind of cell, i.e. total, young and old erythrocytes, we have determined the number of binding sites, the dissociation constants and the RBC populations of high and low affinity.

The results we obtained (Table 1) lead clearly to the following observations.

(i) Young cells possess more sialic acid residues, while ageing *in vivo* reduces this number. This confirms a series of earlier studies showing that the content of surface-bound sialic acid depends on the age of erythrocytes [48, 49, 52–54]. Considering the number of sialic acid-specific lectin binding sites of neuraminidase-treated erythrocytes, on the one hand, and of old erythrocytes, on the other hand, we could conclude that the desialylation of senescent RBCs is low and does not exceed 10% compared to the binding site number for total erythrocytes. This value is in good agreement with results obtained by several authors who assert that removal of as little as 10% of the surface sialic acid of erythrocytes results in their rapid elimination from the circulation [17, 32, 52, 53, 55, 56, 58].

(ii) The number of young and old RBC binding sites for LCA and DSA is not significantly different indicating that there is no loss of α-1,6-fucosyl residues nor of poly-*N*-acetyllactosaminic structures. These results are not in accordance with the conclusions of several authors suggesting that a homogeneous decrease of all of the carbohydrate moieties may occur during ageing of erythrocytes *in vivo* [56–59].

(iii) On the contrary, the experiments carried out with the fucolectins UEA-I and AAA which bind to the blood group O(H) antigen Fuc(α1-2)Gal, indicate an important decrease in the number of binding sites during ageing of O-erythrocytes as previously observed by Shinozuka *et al.* [60] and Sharon and Fibach [50, 51]. In fact the ratio of young RBC receptors to old RBC receptors is 1.21. This ratio is identical to the ratio found by Choy *et al.* [56] using radio-labelled UEA-I in solution. In this regard, it is worthwhile to note that the decrease in the O antigen is comparable to the decrease in cell surface area during ageing of erythrocytes since, according to several authors, the ratio of surface of young RBCs to surface of old RBCs is 1.26. This result could be attributed to a peeling off of membrane vesicles containing O antigen sites and would thus favour the 'vesicle hypothesis'. Consequently, we are tempted to agree with the hypothesis of Aminoff and coworkers [48] who postulated that 'the loss of sialic acid from RBC surface occurs in two forms: (i) as vesicles containing the sialoglycoprotein glycophorin; and (ii) as free sialic acid residues from glycophorin molecules remaining on the cell surface'. The second point of this hypothesis is confirmed by two of our own two observations: (i) in the case of WGA and

MAA, the dissociation constants vary to a large extent as above mentioned and discussed (see Table 1) and, (ii) transfer of radio- or fluorescein-labelled *N*-acetylneuraminic acid by a sialyltransferase is higher on senescent than on young RBCs.

(iv) The finding that the number of binding sites for PNA remains unchanged could lead to the conclusion that the desialylation does not affect the glycophorins which are rich in the sialylated disaccharide Gal(β1-3)GalNAc. This result would be in opposition to authors who claim that the desialylation of glycophorins, of glycophorin A in particular, is responsible for the capture of senescent erythrocytes by macrophage [5, 48, 49, 61]. However, on the basis of our results, we cannot reject the 'glycophorin hypothesis' of Aminoff [9] since the non-reactivity of senescent RBCs with PNA could be attributable to the presence on the erythrocyte surface of the anti-T-autoimmune IgG and IgM present in all human sera according to Aminoff *et al.* [48, 49], Prokop *et al.* [62] and Tanner *et al.* [63]. On the other hand, we must take into consideration the work of Aminoff *et al.* [64, 65] who isolated glycopeptides rich in the disaccharide Gal(β1-3)GalNAc [named Senescence Factor Glycopeptides (SFG)] from tryptic digests of RBCs by affinity chromatography on immobilized PNA. These glycopeptides were detectable in old, but not in young erythrocytes, are free of sialic acid, bind to spleen monocytes (this property being abolished upon treatment with β-galactosidase [64]) and inhibit the adhesion and phagocytosis of senescent RBC by autologous blood monocytes at nanomolar concentrations [65]. In addition, the experiments of Aminoff's group have clearly demonstrated the role of glycophorin in ageing and sequestration of erythrocytes [66] as well as the sequential desialylation of glycophorins in the *in vivo* ageing of erythrocytes, the α-2,6-sialyl residues linked to the disaccharide Gal(β1-3)GalNAc being removed before the α-2,3-linked residues [67].

(v) Curiously, experiments carried out with RCA_{120} and with *Erythrina cristagalli* and *Erythrina corallodendron* lectins led to results which are in contradiction to those described above. In fact, instead of detecting an enrichment in terminal β-galactose residues in old erythrocytes and more in neuraminidase-treated erythrocytes, we obtained the opposite results with a decrease in the number of RCA_{120} and *Erythrina* lectin binding sites associated with increasing desialylation. The same result has been obtained by Shinozuka *et al.* [68] using RCA_{120} and applying the haemagglutination method. To date this result remains to be explained.

However, results of experiments based on haemagglutination with lectins prove that the number of RCA_{120} molecules bound per cell is elevated about three-fold with ageing [56]. In addition, the specific loss of sialic acid residues with the unmasking and exposure of the penultimate β-galactose residues has been demonstrated by several authors by applying the following procedures:

(a) Use of galactose oxidase followed by tritiated borohydride labelling [34, 46, 64, 69, 70].

(b) Transfer of radioactively labelled sialic acid with sialyltransferase leading to the results we described in Table 2 which confirm those of other authors [5, 46, 71].

(c) Transfer of fluorescein-labelled sialic acid with sialyltransferase (this paper).

(d) Increased release of galactose on treatment of old erythrocytes with β-galactosidase [46].

(e) Increase of the reactivity with autoimmune antigalactosyl IgG [72].

(f) Inhibition with β-galactosides of the capture by macrophages of neuraminidase-treated or of old erythrocytes (this paper and [24, 39, 64, 65, 73–78]).

(g) Isolation from old erythrocyte membrane of the senescence factor glycopeptides (SFG) mentioned above [64].

Taking all of the results obtained together, the partial desialylation of erythrocytes during ageing appears as a prerequisite for the phagocytosis of senescent erythrocytes by macrophages. However, according to numerous authors, it seems that the endocytosis of old erythrocytes is a multifactor process which is at least a two-step mechanism comprising initially the β-galactolectin system ensuring the capture of RBCs by macrophage and, secondly, the intervention of natural antibodies. In fact, numerous data strongly suggest that the interactions between old RBCs and macrophages are opsonin-dependent and that the binding to RBCs of autoantibodies of the IgG type ultimately induces erythrophagocytosis by macrophages [4, 8, 11, 12, 15, 47, 77, 79–85]. The C3b macrophage receptor would be part of the machinery [86]. In addition, the role of the cytoskeletal network underlying the RBC membrane which undergoes structural alterations with ageing has been described [49].

The analysis of the abundant literature clearly demonstrates that the problem of the phagocytosis of senescent erythrocytes is not yet solved. However, in the centre of a complex mechanism, the sialic acids play a key role. Assuming this to be correct the following question arises: 'Where is the neuraminidase responsible for the desialylation of erythrocyte membrane glycoconjugates located?'

An initial hypothesis could be based on the mechanism proposed by Bocci *et al.* [18, 87] for explaining enzymatic attacks of erythrocyte membrane components which 'is believed to occur mainly during the fairly short but numerous visits paid by the erythrocytes during their life-time to the spleen and bone marrow where circulation is notoriously sluggish and chances of close contact with granulocytes and macrophages are high'.

A second hypothesis brings into play the neuraminidase present in the membrane of the human erythrocytes themselves and discovered by Bosmann [88]. In fact, as long as this neuraminidase was considered as an integrated enzyme buried in the erythrocyte membrane, it was difficult to envisage that it could act on glycoprotein glycans in positions far from the active site of the enzyme, except for the glycolipids. However, since the demonstration by Tettamanti and co-workers [89]

that the human erythrocyte sialidase is linked to the plasma membrane by a glycosylphosphatidylinositol anchor, the hypothesis of the enzymatic self-desialylation becomes acceptable taking into account the flexibility of the GPI arm allowing the neuraminidase to extend over a large area of the membrane. Consequently, the desialylation would depend on the concept of the probability of an event and not on that of a programmed signal of desialylation.

Conclusion

In conclusion, it is clear that the molecular mechanism of erythrophagocytosis is not yet solved despite the impressive amount of work carried out in this field. However, it becomes more and more evident that the desialylation of erythrocyte membrane glycoconjugates which remain to be identified, is the signal for RBC capture by macrophages and that antibodies play a key role, in a second step. This enigma represents a fascinating and exciting challenge. In addition, as Aminoff *et al.* [2] wrote, 'One of the most extensively studied cells, the red blood cell, with a definite life span, seems to me to be the ideal model to study senescence'.

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