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Role of membrane sialic acid content in the adhesiveness of aged erythrocytes to human cultured endothelial cells

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Following our previous observation that the oldest normal red blood cells were the most adherent to human cultured endothelial cells, we attempted to simulate this age-related adherence. Among all the membrane modifications experienced by erythrocytes during their life-span, loss of sialic acids has attracted considerable attention. Using two different preparations of neuraminidase, we performed a siliac acid depletion on the youngest erythrocytes to reach a sialic acid content similar to that observed in physiologically aged erythrocytes. These pretreated youngest cells displayed limited increase in the adhesiveness to endothelial cells, lower than that found with intact oldest cells. To obtain an adhesiveness of pretreated cells similar to that of naturally aged cells, it was necessary to exceed 80% of sialic acid depletion. At this extent of desialation, modifications of the electrophoretic pattern of glycophorins were observed as well as the appearance of peanut agglutinin reactivity which were never found in physiologically aged erythrocytes. Therefore, the sialic acid loss cannot be considered as being a single determinant factor of the naturally aged red cell adhesiveness.

Introduction

Erythrocyte adhesiveness to endothelial cells had been studied in various pathology and found to be abnormal in sickle cell anemia [1] and diabetes mellitus [2]. The increased adhesivity observed in these diseases were statistically correlated with clinical manifestations [2] and several attempts have been made to explain this phenomena.

We have previously reported that the oldest normal erythrocytes, isolated by density centrifugation, have higher adhesiveness to cultured endothelial cells than the youngest normal cells [3]. In addition, the difference of adhesiveness between young and old red cells was found to be more pronounced in diabetes mellitus, with the oldest diabetic red cells being significantly more adhesive.

Coincidence between red cell ageing and adhesivity prompted us to postulate that modifications experienced by the erythrocytes during their life-span would cause an increase in their adhesivity to endothelial cells.

Among these cell modifications (including reductions in the level of some intracellular metabolites and enzymes [4] and decreases in membrane constituents [5–7]), loss of sialic acid residues has attracted considerable attention. It has been suggested that desialation by itself may play a role in

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the sequestration of erythrocyte from circulation [8–12].

In order to determine whether desialation plays a role in the adhesiveness of old cells to endothelium, we studied the adhesiveness to endothelial cultured cells of red blood cells naturally depleted of sialic acids (old cells isolated by density) and young red blood cells artificially depleted of sialic acids by neuraminidase treatment. Erythrocyte glycophorins were concomitantly studied after separation by electrophoresis and staining by periodic acid Schiff reagent. Reactivity with peanut agglutinin was also investigated after electroblotting of the separated glycoproteins.

Materials and Methods

Separation of erythrocyte fractions

Red blood cells were separated by density centrifugation through a discontinuous gradient of stractan as originally described by Corash et al. [13]. Prior to its use, stractan solution (Sigma stractan II purchased from Mallet SA, Roissy, France) was deionized with Bio-Rad's analytical grade ion exchange resin (AG 501-X8 purchased from Touzart et Matignon, Vitry/Seine, France). For erythrocyte separation, 5 bands of stractan solutions (densities 1.120 (5 ml); 1.100 (8 ml); 1.090 (8 ml); 1.080 (5 ml)) were layered on each other in a 25×89 mm ultra-clear tubes (Beckman-France, Gagny). 2 ml of three-times washed and packed erythrocytes were layered per tube and centrifuged for 1 h at $100000 \times g$ in a Beckman SW 27 rotor. Four erythrocyte fractions were obtained and are referred to as fraction 1 to fraction 4, containing, respectively, erythrocytes of the lightest to the densest population.

For each erythrocyte fraction, routine hematological determinations (including the mean corpuscular hemoglobin concentration) were done using Coulter Counter Model S + IV. Reticulocyte counts were performed after new methylene blue staining.

Preparation of erythrocyte membranes (ghosts)

Each isolated erythrocyte fraction was extensively washed in 5 mM sodium phosphate, 150 mM NaCl (pH 8.0). The ghosts were prepared according to the method of Litman et al. [14] with 0.6 mM phenylmethylsulfonyl fluoride and 1 mM ethylenetetraacetic acid added to all buffers.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis were performed as described by Laemmli [15]. The running gel consisted in a 9% polyacrylamide gel and the gels were stained by Coomassie blue as reported by Fairbanks et al. [16].

Membrane glycoproteins (glycophorins A, B and C) were stained with periodic acid Schiff as reported by Konat et al. [17].

Labelling of peanut agglutinin with iodine-125

Peanut agglutinin (purchased from Industrie Biologique Française, Villeneuve la Garenne, France) was labelled with iodine-125 as described by McGregor et al. [18] using the Chloramine T procedure.

Study of peanut agglutinating reactivity on electrotransfer blot

Red cell membranes (50 μg of whole proteins per lane of electrophoresis) were electrophoresed on a 9% SDS-polyacrylamide gel. Proteins were electrophoretically blotted onto a nitrocellulose sheet (0.45 μm pore size, Schleicher and Schull purchased from Cera Labo, Aubervilliers, France) as described by Towbin et al. [19]. Blotting was performed for 3 h at 0.22 A, 55 V using a Transblot cell (Bio-Rad laboratories).

The nitrocellulose filters were soaked for 1 h at 37° C and then overnight at 4° C in 5 mM sodium phosphate, 150 mM NaCl (pH 8.0) containing 1.5% bovine serum albumin. After 2 h incubation at room temperature with labelled peanut agglutinin in 5 mM sodium phosphate, 150 mM NaCl, pH 8.0 (600000 cpm/ml; 5 ml for 6 lanes of electrophoresed and blotted membrane proteins), the membrane filters were washed six times for 10 min in 5 mM sodium phosphate, 150 mM NaCl, pH 8.0 and then dried. Autoradiographies were done by exposing nitrocellulose sheets to a Kodak X-OMAT MA film (Eastman Kodak Co, purchased from Euromedica, Paris, France) for 20 h.

Neuraminidase treatment of intact erythrocytes

The youngest and oldest red cells separated by density centrifugation were washed and suspended (20% hematocrit) in a 5 mM phosphate buffer (pH 8.0), containing 130 mM NaCl, 20 mM KCl and 5.4 mM glucose (phosphate-glucose buffer). Two preparations of neuraminidase were used: from *Clostridium perfringens* (purchased from Boehringer Mannheim, Meylan, France, 3 U/mg at 37 °C) and from *Vibrio cholerae* (purchased from Hoechst-Behring, Rueil-Malmaison, France). The enzyme was dissolved in a Tris-acetate buffer (pH 5.7), containing 0.3 mg/ml of bovine serum albumin (stock solution of 1 mg/ml). After appropriate dilution of neuraminidase stock solution in the Tris-acetate buffer, the enzyme was added to red cell suspension (20% hematocrit) at the concentrations mentioned in the text and incubation was performed for 1 h at 37 °C. The reaction was stopped by putting test tubes in ice and diluting 20 times the cell suspensions with cold phosphate-glucose buffer. Three washes with the same buffer were made before preparing membrane ghosts as described above.

Sialic acid determination

Total sialic acid content of erythrocyte ghosts (intact ghosts or after enzymatic depletion of red cells) was determined following incubation of membrane at 80 °C for 1 h with an equal volume of 0.1 M H_2SO_4. The thiobarbituric acid method of Warren [20] was used and all determinations were made in duplicates.

Results were expressed as nanomoles of sialic acid per mg of whole membrane proteins, allowing us to calculate the percentage of sialic acid depletion.

Red cell adhesivity to human cultured endothelial cells

The adhesiveness of erythrocyte was measured as previously described [21]. Briefly, endothelial cells from human umbilical veins were harvested and grown to confluence in 35 mm plastic dishes. Washed erythrocytes were labelled with ^{51}Cr and resuspended to 25% hematocrit. Labelled red cells were layered on primary cultured endothelial cells. After 30 min incubation at 37 °C, the non-attached cells were removed by aspiration and the cultured plates were rinsed five times with 1 ml aliquots of Hanks' balanced salt solution with 5 g of albumin per liter. Adhesiveness was expressed as the number of red cells that remained in the culture after the fifth wash. When the adhesivity of the cells pretreated with neuraminidase was tested, it was compared to that of the same red cells, incubated in the same enzyme buffer for the same period of time, but in the absence of neuraminidase.

Results

The reality and the reproducibility of cell separations were supported by the reduction of the reticulocyte count from the lightest to the densest fraction and by the concomitant increase in the mean corpuscular hemoglobin concentration (Table I). In the experiments we described in this paper, two fractions were used, fraction 1 and fraction 4, referred to as the youngest and oldest cells, respectively.

As previously reported [3], we observed that the oldest red cells were significantly more adherent ($P < 0.01$) than the youngest ones to cultured human endothelial cells (see legend of Table II). Using Warren's method [20], we estimated that the oldest erythrocytes contain 24% less sialic acids than the youngest cells. This mean value is similar to those reported by many other authors [6,8–12] who found a physiological depletion of membrane bound sialic acids ranging between 10 and 20%. Erythrocyte membranes prepared from each fraction of separated cells were also studied for their electrophoretic glycophorin pattern and for peanut agglutinin reactivity. We observed no modification of the glycophorin pattern and no peanut agglutinin reactivity in naturally aged red blood cells (data not shown).

The youngest red cells were then pretreated with different concentrations of neuraminidase and their adhesivity to cultured endothelial cells was tested and compared to that of intact young and old cells isolated in the same time (Table II).

Two neuraminidase preparations (*C. perfringens* and *V cholerae*) were used to perform partial removal of sialyl residues from the youngest red cells to an extent (16% and 20%1 respectively) which mimics the loss of sialic acids during red

TABLE I

CONTROLS OF RED CELL SEPARATION BY DENSITY CENTRIFUGATION

Four fractions of red cells were isolated from the top (fraction No 1) to the bottom (fraction No 4) of the discontinuous gradient of stractan n, number of experiments

Isolated fractions	Mean ± S E (%)			
	1	2	3	4
Reticulocyte counts ($n = 5$)	29±08	18±04	14±03	04±02
MCHC [a] ($n = 5$)	343±05	367±05	402±15	424±34
Amount of recovered cells [b] ($n = 7$)	66 ±45	254±3	61±13	23±07

[a] Mean corpuscular hemoglobin concentration

[b] 100% is equal to the summation of cell number found in each fraction

cell ageing in vivo. As shown on Table II, a slight and non-significant increase in adhesivity was found after treatment with *V cholerae* neuraminidase; with *C perfringens*, red cell adhesiveness increased to a greater extent although it did not reach the level of statistical significance. No modification of glycophorin pattern and no peanut agglutinin reactivity were found in these partially depleted red cells (data not shown).

For the youngest red cells to achieve an adhesiveness level similar to that of the oldest ones, it was necessary to perform a sialic acid depletion higher than 80% (Table II) The oldest red cells, treated with the same amount of enzyme in order to reach 75% sialic acid depletion, did not display any more increase in adhesivity to endothelial cells ($520 \cdot 10^3$ cells per dish)

After quasi complete sialic acid depletion, youngest red cells displayed alterations of their glycophorin pattern (Fig. 1), with an enzymatic concentration-dependent reduction of periodic acid Schiff staining and a shift of more blunted bands to positions of higher electrophoretic mobilities. These modifications were easily observed for the principal glycophorin, the glycophorin A, as previously described by Dzandu et al. [22], using a silver staining procedure. The higher mobilities corresponded to the expected decrease in molecular weight after release of sialic acids [22]. As reported by Dzandu et al. [22], our treatment of red cells with neuraminidase did not modify the membrane protein pattern after staining with Coomassie blue. These modifications of the periodic acid Schiff staining pattern were accompanied with the appearance of peanut agglutinin reactivity at the same migration levels as the peri-

TABLE II

COMPARISON BETWEEN THE ADHESIVENESS AND THE SIALIC ACID CONTENT OF YOUNG RED CELLS, OLD RED CELLS AND YOUNG RED CELLS TREATED WITH NEURAMINIDASE

Adhesiveness is expressed as the number of red cells remaining after the fifth wash The predetermined erythrocyte adhesiveness was for the youngest cells 232 (±140)·10^3 cells/dish (12 experiments ± S D) and for the oldest cells 570 (±210) 10^3 cells/dish (9 experiments ± S D) Two preparations of neuraminidase from *Clostridium perfringens* and *Vibrio cholerae* were used to deplete young erythrocytes The amount of enzyme for 500 µl of packed cells was indicated in mU All the data shown in this table were the mean of two experimental determinations except for those marked with an asterisk (one determination)

Adhesiveness youngest erythrocytes (10^3 cells/dish)	Adhesiveness of pretreated youngest erythrocytes (10^3 cells/dish)	Induced sialic acid depletion (%)	Adhesiveness of oldest erythrocytes (10^3 cells/dish)
176	280 (*C perfringens* 2 mU)	16 *	520
160	190 (*V cholerae* 1 mU)	20 *	480
160	600 (*C perfringens* 250 mU)	88	560

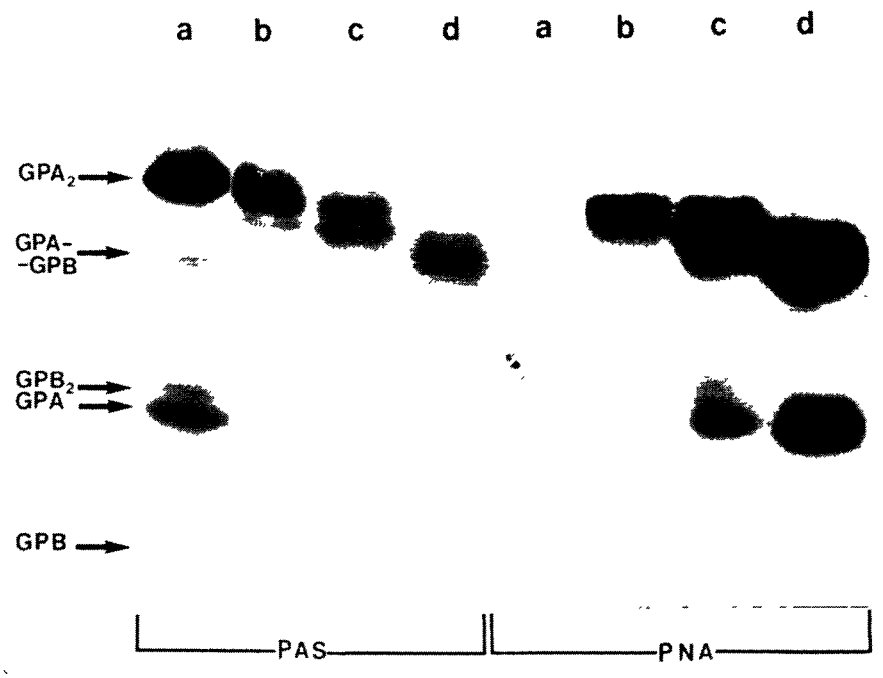

Fig. 1 Glycophorin electrophoretic pattern and peanut agglutinin reactivity after treatment of red blood cells with neuraminidase PAS, polyacrylamide gel electrophoresis of erythrocyte membranes stained with the periodic acid Schiff reaction PNA, after electrophoresis, the polyacrylamide gel was blotted onto nitrocellulose sheet which was probed with ^{125}I-labelled peanut agglutinin The positive picture of autoradiography was shown a, intact erythrocytes membranes, b, c and d, erythrocyte membranes depleted of sialic acids (b 60%, c 80%, d 90%). GPA, glycophorin A, GPB, glycophorin B

odic acid Schiff stained bands. This peanut agglutinin reactivity increased with the depletion of sialic acids (Fig. 1).

Discussion

The limited increase in youngest erythrocyte adhesiveness after partial sialic acid depletion by different neuraminidases did not reach the extent of adhesivity observed with the oldest red cells and appeared to be insignificant. Hebbel et al. [1], in order to explore abnormal adherence of sickle cells to cultured endothelium, carried out partial enzymatic depletion of the whole population of normal red cells by neuraminidase and found no increase in the adhesivity of depleted cells. So, no relationship between partial depletion of sialic acids and adhesiveness to endothelial cells could be established. However, artificial and complete depletion by neuraminidase can significantly increase the adhesivity to endothelial cells. Similarly, Makler [23] has shown that *Plasmodium falciparum* produces a neuraminidase-like activity on invasion into red cell which enhances the binding capabilities of the red cell to the host's endothelial cells. These non-physiological processes of desialation, using added neuraminidase activities, could be completely unrelated to the natural depletion of sialic acids. Artificial depletion of sialic acids by neuraminidase could be also less effective since the bacterial enzymes have a less discriminant action. During red cell ageing, in addition to loss of membrane portions containing intact sialoglycoproteins [11,12], a real desialation of glycoproteins with exposure of the penultimate sugars galactose and *N*-acetylgalactosamine was described [24]. A complete depletion of sialic acids could be necessary to unmask all the few specific sites effectively involved in cell adhesion.

The studies reported by Skutelsky et al. [25] on the relationship between sialic acid content and peanut agglutinin binding on senescent and enzyme-treated erythrocytes have also indicated that the age related loss of sialic acids is not identical

with the enzymatic removal by neuraminidase. In agreement with our results but using different methods, these authors found that intact old cells, separated by differential flotation, and displaying about 20% reduction of sialic acids, did not agglutinate with peanut agglutinin and failed to bind labelled peanut agglutinin. Conversely, after treatment with neuraminidase resulting in 10% reduction of sialic acids, young red cells did agglutinate with peanut agglutinin. Concomitantly, about 10 binding sites for peanut agglutinin-ferritin/μm of sectioned membranes were revealed and 26 binding sites when the depletion of sialic acids reached 60%. In our case, it was necessary to remove more than half the sialic acids (obtained with 75 to 100 mU of neuraminidase/500 μl of packed cells) to get peanut agglutinin reactivity as well as specific modifications of the glycophorin pattern.

Many studies concerning red cell membrane alterations, thought to be involved in the recognition of senescent cells, raised doubts about a single role for desialation [26]. While treatment of red cells with neuraminidase shortens cell survival [27], it does not always result in a rapid clearance of the desialated cells [28], as might have been expected if loss of sialic acids was the primary signal for cell removal. As we observed in the interaction between senescent red cells and endothelial cells, desialation could be an helper or encouraging factor rather than a determinant signal for the trapping of old cells.

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