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CHANGES IN SURFACE CARBOHYDRATES OF ERYTHROCYTES DURING *IN VIVO* AGING

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SUMMARY: Erythrocyte aging is accompanied by an overall reduction in surface carbohydrate content. A decrease in sialic acid and an increase in D-galactose and N-acetylgalactosamine in the terminal position of the glycoprotein polysaccharide chains are also observed in aged erythrocytes. In the light of these and other observations, it is proposed that the newly exposed galactose/galactosamine residues in the desialylated glycoproteins may serve as recognition signals triggering the elimination of senescent erythrocytes from circulation.

INTRODUCTION

The cell surface plays a crucial role in many important biological functions, including infection interaction, embryonic development, immune response to foreign antigen and clearance of senescent cells. Complex carbohydrates are thought to be highly significant in this respect by conferring specificity to structures in which they occur. For example, the presence of sialic acid on the cell surface has been shown to be crucial for the survival of mammalian erythrocytes in circulation (1, 2). Neuraminidase-treated mammalian erythrocytes are removed from the circulation significantly faster than intact erythrocytes (3,4). In order to understand more of the physiological mechanism of erythrocyte senescence and factors affecting their clearance, we studied the surface carbohydrates of young and old erythrocytes.

MATERIALS AND METHODS

Purified lectins, wheat germ agglutinin (WGA), Ricinus Communis agglutinin II (RCA-II), Bauhinia Purpurea agglutinin (BPA), Ulex Europaeus agglutinin I (UEA-I) and Concanavalin A (Con A) were obtained from E-Y Laboratories (San Mateo, U.S.A.) and ^{125}I (100 m Ci/ml) was supplied by Amersham Searle. *Vibrio cholerae* neuraminidase was a product of Calbiochem.

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Separation of erythrocytes of different ages Each blood sample, freshly drawn from normal donors, was collected into heparinized tubes and treated separately. Human erythrocytes of different ages were separated on the basis of density differences by ultracentrifugation (67,000 g, 2 hrs, 4°C) according to the method of Rigas *et al.* (5). The top and the bottom layers (each representing 5-10% of the total volume) of the packed red cells were removed by gentle aspiration and assayed for the difference of ages by comparing the activities of the enzyme markers — glucose-6-phosphate dehydrogenase (6) and cholinesterase (7) activities.

Lectin binding studies The five purified plant lectins were radio-labelled with ^{125}I by the rapid chloramine T exposure technique of Hunter (8, 9). Lectin binding assays by microcentrifugation at 0°C were performed according to the methods of Speckart (10) with suitable inhibitors for the lectins and corrected for non-specific binding. The number of lectin molecules bound/cell was determined according to the method of Steck and Wallach (11).

Analysis of cell surface carbohydrates Young and old erythrocyte membranes were prepared according to the method of Hanahan *et al.* (12). For determination of neutral sugars, the sample was hydrolyzed with 3 N HCl at 100°C for 90 min (13). The released neutral sugars were then analysed as their alditol acetates (14) by gas-liquid chromatography, using a Varian gas chromatograph (Model 3700, equipped with flame ionization detectors), stainless steel column (84" x 1/8") filled with 3% ECNSS-M on Gas-chrom Q (100/120 mesh), and at a column temperature of 175°C. Hexosamines were released by the same conditions. Their derived alditol acetates were again analysed by gas-liquid chromatography using a column packed with 3% poly(A) 103 on Gas-chrom Q (100/120 mesh) at 230°C (15).

Sialic acid was assayed by the thiobarbiturate method of Warren after the sample was hydrolysed with 0.05N sulfuric acid at 80°C for 1h (16).

Statistical evaluation of data was carried out using Student's t-test. Significant differences between the means (calculated as p values) are shown when appropriate.

RESULTS

Marker enzyme studies (Table 1) indicate that glucose-6-phosphate dehydrogenase and cholinesterase activities are both significantly lower in cells from

Table 1. Separation of Human Erythrocytes of Different Ages by Centrifugation

	Glucose-6-phosphate dehydrogenase[a]	Cholinesterase[b]
top layer	1.7 ± 0.24	21.2 ± 3.0
bottom layer	0.83 ± 0.034	6.33 ± 0.03
p[c]	<0.001	<0.001

a. Results are expressed in units/10^{10} cells as mean ± S.D. One unit of enzyme activity reduces 1 μmole of NADP under the specified conditions (6)

b. Results are expressed in units/10^{10} cells as mean ± S.D. One unit of enzyme activity hydrolyses 1 μmole of acetylthiocholine/min. at 25°C, pH8.0(7)

c. p value for difference between top and bottom layer cells

Table 2. Carbohydrate Analysis of Erythrocyte Membranes.

Carbohydrate	Young Cells n mole/mg protein	Old Cells n mole/mg protein	p Values
L-Fucose	65.48 ± 4.2	53.71 ± 4.5	<0.01
D-Mannose	41.87 ± 3.5	29.1 ± 2.7	<0.002
D-Galactose	389.3 ± 16	311.4 ± 11	<0.001
D-Glucose	61.67 ± 4.3	46.1 ± 3.4	<0.001
D-Glucosamine	64.5 ± 5.2	49.3 ± 2.7	<0.005
D-Galactosamine	48.4 ± 3.1	37.8 ± 2.4	<0.002
Sialic acid	96.7 ± 3.8	83.2 ± 3.7	<0.005

the bottom layer than those in the top. This demonstrates that the bottom layer contains preponderantly old cells while the young cells are concentrated in the top layer (5). Table 2 shows the results of analyses of surface carbohydrates in erythrocytes. D-galactose is the predominant sugar, constituting about 50% of the total carbohydrates. It is also seen that erythrocyte aging is accompanied by a reduction of carbohydrate content in the cell membrane. The reduction was observed in all seven sugars examined and ranged from 14% for sialic acid to 30% for D-mannose.

The abilities of erythrocytes to bind lectins of various sugar specificities are summarised in Table 3. At once apparent is that old erythrocytes, untreated

Table 3. Lectin Binding by Erythrocyte

Lectin	Sugar Specificity	Lectin molecules bound/cell x 10^{-6} (±S.D.)[a]			
		Untreated		Neuraminidase-treated	
		young cells	old cells	young cells	old cells
WGA	D-Glc NAc sialic acid	16.7 ± 2.12	10.1 ± 0.87	13.0 ± 2.9	8.5 ± 1.4
RCA-II	β-D-gal D-gal NAc	0.75 ± 0.15	2.1 ± 0.41	4.76 ± 0.54	3.96±0.62
BPA	D-Gal NAc	0.24 ± 0.13	0.68±0.27	1.41 ± 0.35	1.15±0.47
UEA-I	α-L-Fucose	0.57 ± 0.14	0.47±0.08	0.61 ± 0.36	0.49±0.14
Con A	α-D-Man α-D-Glc	0.30 ± 0.08	0.20±0.02	0.46 ± 0.12	0.21±0.06

[a] Molecular weights of lectins used for calculation are: WGA 36,000 (21,22) RCA-II 60,000 (23) BPA 195,000 (24) UEA-I 70,000 (25) Con A 110,000 (26).

with neuraminidase, carry significantly fewer receptors for WGA compared with young ($p<0.001$). By contrast, the number of binding sites for RCA-II and BPA are elevated about 3-fold with aging ($p<0.001$) while those for UEA-I and Con A are relatively unaffected.

Treatment with neuraminidase greatly enhances the ability of both young and old cells to bind RCA-II and BPA ($p<0.02$ for all comparisons made) but has no effect on the cells' capacity to bind UEA-I and Con A. As expected, the number of receptors for WGA is reduced by neuraminidase.

DISCUSSION

The removal of sialic acid from membrane glycoprotein has been proposed to be a major factor in the elimination of old erythrocytes from circulation (1,3, 4). The observation by Aminoff (17) that only neuraminidase-treated rat erythrocytes could form rosettes with isolated liver macrophages and spleen mononuclear cells and by Kolb (18) that this rosette formation is selectively inhibited by N-acetyl-D-galactosamine, D-galactose and fucose but not by other sugars would seem to suggest that it is not desialylation *per se* but the consequent exposure of the penultimate sugar that is the triggering event.

Our present study indicates that while D-galactose constitutes about 50% of the total carbohydrate in the erythrocyte membrane, sialic acid is the predominant terminal sugar in the glycoprotein polysaccharide chain. The observation that treatment with neuraminidase does not alter the binding of UEA-I and Con A by both young and old erythrocytes demonstrates that the penultimate sugar is unlikely to be α-L-fucose, α-D-mannose or α-D-glucose. The decrease in WGA binding and the 3-fold rise in receptor sites for RCA-II and BPA in old erythrocytes compared with young may be interpreted to mean that during the cell's life span, sialic acid is removed from the surface sialoglycoproteins, thus exposing the subterminal galactose or galactosamine residues. These may then serve as recognition signals to initiate cell clearance, as suggested by the work of Kolb (18). The possibility that these galactose and galactosamine are newly synthesized residues may be ruled out as far as mature erythrocytes are concerned (19).

Our experiments with neuraminidase show that even in aged erythrocytes a considerable amount of sialic acid-galactose- and sialic acid-galactosamine-termini remains, as can be seen from the further 2-fold increase in RCA-II and BPA binding following enzyme treatment. The cell clearance event, if indeed triggered by galactose/galactosamine exposure, must be a threshold event, precipitated by the unmasking of less than 50% of these penultimate sugars. This proposal is consistent with the observation (1) that removal of as little as 10% of the surface sialic acid from erythrocytes results in their rapid elimination from circulation and is akin to Ashwell's model (2) for the clearance of serum glycoproteins.

It is not clear how or why carbohydrates are lost from erythrocyte membranes (Table 2) during the aging process. Prankerd (20) suggested that segments of membranes might have been pinched off as erythrocytes are squeezed through small vascular apertures. Regardless, the decrease in surface carbohydrates may provide an alternative mechanism for unmasking previously cryptic receptors as a result of changes in membrane structure.

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