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SURFACE CARBOHYDRATES OF AGED ERYTHROCYTES

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Summary. Human erythrocytes, fractioned into populations of different density by ultracentrifugation in albumin gradients were examined to determine what changes in cell surface carbohydrates occur during their life-span. In addition to changes occurring in N-acetylneuraminic acid ageing was accompanied by reduction in the N-acetylglucosamine, N-acetylgalactosamine and galactose content of erythrocyte membranes. These results show that extensive heterogeneity exists in the cell surface carbohydrate of the circulating population of erythrocytes and suggest clearance of neuraminidase treated erythrocytes may not be an adequate model for the removal of aged cells.

Mature human erythrocytes survive in the circulation for a period of about 120 days before aged cells are selectively removed in the reticulo-endothelial system by a non-random mechanism(1). During in vivo ageing many changes in chemical and physical properties occur but it is not known which, if any, of these changes is directly related to the recognition and removal of aged erythrocytes.

Because of the surface location of membrane glycoproteins and glycolipids and the role of complex carbohydrates in cellular recognition processes there has been interest in the possibility that these substances might be implicated in the circulatory survival of red blood cells. Removal of sialic acid by treatment with neuraminidase (2) results in rapid removal of erythrocytes from the circulation suggesting that exposure of subterminal carbohydrate residues might be necessary for this process to occur (3).

In this study membranes from human erythrocytes of different in vivo age have been examined to determine whether other changes in cell surface carbohydrate occur and whether these could be related to circulatory survival.

METHODS

Fresh blood was obtained by vene-puncture and was mixed with one tenth

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volume of Alsever's solution. Plasma, white cells and platelets were aspirated after centrifugation at 800g for 10 min, and the cells were washed four times in 5mM NaH_2PO_4 buffer containing 0.15M NaCl adjusted to pH 7.4 with NaOH (PBS 7.4). Haemoglobin free erythrocyte membranes were prepared by lysis and washing in 5mM sodium phosphate buffer pH 8.0 (4). However the 'button' of material packed at the bottom of the ghost pellet was found to consist of membranes from the oldest cell fractions and was not discarded.

Bovine albumin, Fraction V (Sigma) was deionised, lyophilised and then redissolved in buffer of composition of 153mM $NaHCO_3$, 5.2mM $MgCl_2$ and 5mM KCl, containing 0.1 mg/ml streptomycin sulphate and 0.6 mg/ml penicillin, to give a protein concentration of approximately 42% (w/w) and the osmolarity was adjusted by addition of solid NaCl to 290 milliosmoles/kg. Fractions of the required specific gravity and having pH 7.4 were obtained by dilution of the concentrated albumin with a solution containing 145 mM NaCl, 5.5mM $MgCl_2$ and 5mM KCl. Washed, packed erythrocytes (1 ml) were applied to the top of a discontinuous albumin density gradient. Gradients consisted of five 2.3 ml layers ranging in density from 1.115 g/ml (measured at 20°C) with decreasing steps of 0.004 - 0.006 g/ml and were centrifuged at 4°C for 40 minutes in a Beckman SW40 rotor at 40,000 r.p.m. The six fractions obtained were washed six times in PBS 7.4. The distribution of cells among the fractions was obtained by cell counting and by haemoglobin determination.

For agglutination experiments serial dilutions of anti-A or anti-B blood group sera (Hyland) were prepared in the wells of a plastic agglutination tray and to each well was added 0.5 volumes of 1.5% (v/v) erythrocytes which had been fractionated on albumin gradients. Agglutination was allowed to proceed for 30 min at 20°C.

N-acetylneuraminic acid was released from erythrocyte ghosts by hydrolysis in 0.05M H_2SO_4 at 80°C for 1 h, partially purified on a column of Dowex 1 x 8-100 formate, and estimated colourimetrically (5).

Aminosugars and amino acids were determined after hydrolysis in 4M methane sulphonic acid in evacuated sealed tubes for 70 h at 100°C. Neutral sugars were determined by g.l.c. (6).

Galactose and N-acetylgalactosamine residues on cells were labelled by the method of Gahmberg & Hakomori (7).

Electrophoresis of membrane preparations was performed as described by Fairbanks *et al.* (4) except that samples were heated at 100°C for 3 min before application to the gels.

RESULTS

Each fraction contained 10-20% of the original cell population. When isolated fractions were reapplied to a new gradient and recentrifuged 75 - 80% of the cells could be recovered from their original fraction. Small numbers of white cells (approximately 0.1% of all cells) and reticulocytes were detectable in gradient fraction 1 (which contained approximately 10% of the youngest red cells) but no cells other than erythrocytes were observed in the more dense fractions. For this reason analyses were related to values

observed in fraction 2.

The activity of glucose-6-phosphate dehydrogenase declined in activity per cell by 79% between fraction 2 and the most dense cell fraction 6.

A progressive increase in the rate of agglutination of more dense cell fractions was observed with anti-A and anti-B sera. The difference in dilution of antiserum producing a similar extent of agglutination between youngest and oldest cells was marked (3-4 serial dilutions).

The profiles obtained on SDS-gel electrophoresis of membranes isolated from young and old cell fractions labelled using galactose oxidase and tritiated sodium borohydride showed minor differences but no substantial increase in labelling was observed corresponding to components PAS 1 or PAS 2 (Fig. 1). Thus cell ageing does not appear to be accompanied by detectable unmasking of galactose or N-acetylgalactosamine residues in glycophorin.

The average carbohydrate content of membranes from cell fractions 2, 4 and 6 for three individuals is shown in Table 1.

Pronounced changes occur in glucosamine, galactosamine and galactose which were lower by 28%, 33% and 33% respectively in the oldest cell fraction (G6) when compared to fraction G2.

The carbohydrate analyses of membrane fractions were related directly to the content of the amino-acids obtained in the same analysis as the amino sugars. The amino acid compositions of all cell fractions were similar. SDS-gel electrophoresis of membranes showed that the proportion of major membrane proteins were similar in young and old cells.

DISCUSSION

Labelling studies have established that the specific gravity of erythrocytes increases on ageing (8). Centrifugation in density gradients of albumin allows separation of cell fractions of defined density which show marked differences in enzymatic activity and agglutination properties presumably related to *in vivo* ageing.

It has previously been established that there is a reduction in the

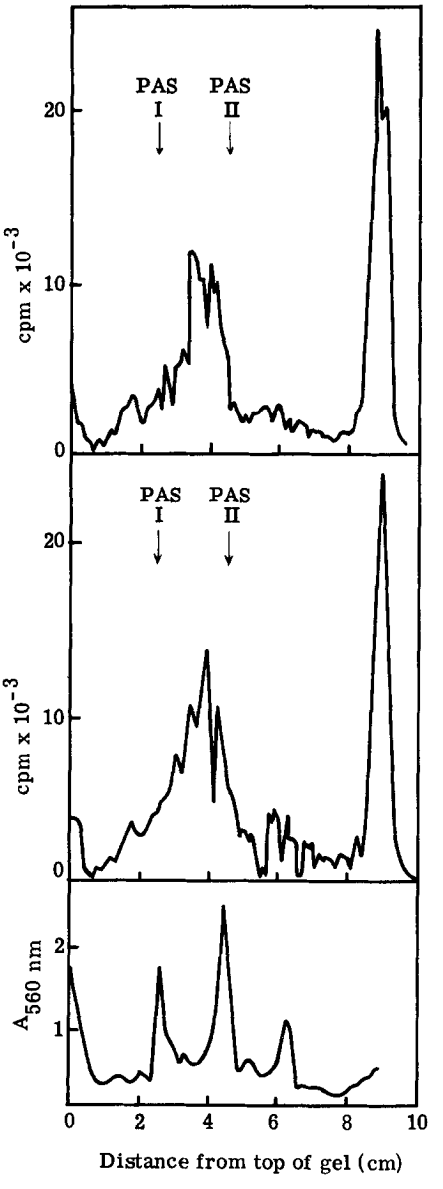

Fig. 1. Labelling of Galactose and Galactosamine Residues of Erythrocyte Membrane Glycoproteins and Glycolipids

Tritium was incorporated into galactose and galactosamine residues of membrane glycoproteins and glycolipids of (a) young (fraction 2) and (b) old (fraction 6) erythrocytes by treatment with galactose oxidase followed by (^{3}H)-borohydride reduction. Isolated membranes were subjected to SDS/polyacrylamide gel electrophoresis and gel slices were counted. A control gel (c) was stained with periodic acid - Schiff reagent and scanned at 560nm to reveal the sialoglycoproteins. Positions of PAS I and II (glycophorin) are indicated by arrows.

Table 1 Carbohydrate content of membranes obtained from erythrocyte fractions of different in vivo age.

	mol of carbohydrate residues/100 mol of amino acid residues			
	$GlcNH_2$	$GalNH_2$	Gal	NANA
G2	1.10 ± .14	0.78 ± .16	3.22 ± .14	0.90 ± .16
G4	1.14 ± .19	0.75 ± .16	2.54 ± .28	0.78 ± .18
G6	0.80 ± .09	0.52 ±0.17	2.16 ± .25	0.75 ± .16

Fraction G6 contains membranes from the 10% most dense cells, G2 represents the lightest fraction (approximately 10% of cells) not contaminated by reticulocytes and G4 (approximately 20% of cells) was obtained from cells of intermediate density.

Analyses were carried out on blood from three individuals and are given ± standard deviation.

sialic acid content of the oldest, most dense, fractions of the erythrocyte population (5,9). The results reported here show that changes in other carbohydrate residues also occur, especially in galactose and N-acetylgalactosamine. Loss of these residues provides an explanation for the decreased binding of soybean agglutinin by old cells when compared to young cells (10).

Treatment of erythrocytes with neuraminidase results in greatly increased labelling of glycophorin using the galactose oxidase tritiated borohydride procedure (7). If loss of sialic acid from aged erythrocytes results from neuraminidase action increased exposure of subterminal galactose in old cells would be predicted. However labelling of exposed galactose and N-acetylgalactosamine residues of young and old cell fractions did not indicate increased labelling of glycophorin (Fig. 1). This observation is consistent with the finding that old cells, unlike neuraminidase treated cells, do not show an increase in binding of peanut agglutinin (11). Thus studies of erythrocytes which have been extensively treated with neuraminidase may not provide a good model for the physiological process of removal of aged erythrocytes from the circulation.

Although it is clear that there is considerable heterogeneity in the surface carbohydrate in the circulating population of red blood cells it is

not known how this arises. Possibilities requiring investigation include loss of intact membrane glycoproteins or glycolipids, proteolytic cleavage and loss of carbohydrate by the combined action of glycosidases.

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