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# *In vivo* AGEING OF HUMAN ERYTHROCYTES AND CELL-SURFACE LABELING BY D-GALACTOSE OXIDASE AND SODIUM BOROTRITIDE

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## ABSTRACT

Young and old, human erythrocytes, separated *in vitro* according to their age *in vivo*, were radioactively labeled at the cell-surface D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues by treatment with D-galactose oxidase, followed by reduction with sodium borotritide. The labeling was quantitatively determined for each type of erythrocyte by measuring the molar amounts of borohydride necessary for the complete reduction of the oxidized residues. The number of surface residues per blood-group $A^+$ erythrocyte was found to be $37.6 \pm 1.8 \times 10^6$ ($n = 8$) for young, $21.8 \pm 4.9 \times 10^6$ ($n = 8$) for old, and $24.8 \pm 6.4 \times 10^6$ ($n = 8$) for middle-aged erythrocytes, indicating a significant decrease of the residues during ageing.

## INTRODUCTION

We have previously shown that a significant decrease of each of the seven carbohydrate components of the membrane of human erythrocyte occurs during *in vivo* ageing[1,2]. The content of carbohydrate of the old erythrocyte is ~87% of that of the young one. However, the analysis of the whole membrane does not reflect the changes occurring at the surface of the cell during ageing[1], and the D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues may be involved in the physiological clearance of the old erythrocytes. To determine the number of these residues at the surface of young and old human erythrocytes, the cells were separated *in vitro* according to their age *in vivo*, and then labeled by treatment with D-galactose oxidase. This was followed by reduction with sodium borotritide, which was quantitatively determined. The number of residues per erythrocyte was calculated (a) by the use of an experimental quenching curve that was obtained with various amounts of erythrocytes and a constant level of isotope, and (b) by subtracting for each experiment the nonspecific labeling of the cells treated under identical conditions, but without D-galactose oxidase. The chemiluminescence of the hemoglobin of erythrocytes was decreased by addition of an appropriate amount of hydrogen peroxide, and the nonspecific labeling was decreased by use of an antiproteolytic compound (phenylmethylsulfonyl fluoride).

EXPERIMENTAL

*Separation of erythrocytes of different ages.* — Each blood sample, freshly drawn from normal donors of $A^T$ blood-group, was collected into heparinized tubes and treated separately. As the density of erythrocytes increases with ageing[3], the erythrocytes of different ages were separated by centrifugation for 2 h at 2000*g* at 18°, as previously described[1,2]. In each experiment, young, old, and middle-aged erythrocytes were prepared.

*Labeling of young, old, and middle-aged A erythrocytes.* — The labeling of the cells was performed as described by Gahmberg *et al.*[4–6] with slight modifications in the use of different molar amounts of sodium borotritide, in order to determine for each type of cells (young, old, or middle-aged) the amount of sodium borotritide necessary for the complete reduction of the D-galactose-oxidized residues. The erythrocytes were washed four times with phosphate-buffered saline solution (PBS) containing 20mM sodium phosphate and 0.12M sodium chloride (pH 7.4). The washed erythrocytes ($8 \times 10^9$ cells/mL) were incubated for 30 min at 37° in PBS (2 vol.) containing D-galactose oxidase (5 units, Worthington Biochemical Corp., Freehold, NJ 07728; or Sigma Chemical Co., St. Louis, MI 63178) per mL of packed erythrocytes and 0.2mM phenylmethylsulfonyl fluoride (Sigma). To determine, in each experiment, the nonspecific labeling of the cells, aliquots of young, old, and middle-aged erythrocytes were incubated in the buffer without D-galactose oxidase.

After being washed thrice, the samples were reduced for 30 min at room temperature with various amounts of sodium borotritide, obtained either from New England Nuclear (D-6072 Dreieichenhain, West Germany) or from the Radiochemical Centre (Amersham France, F-78005 Versailles, France). The sodium borotritide was added to the PBS just before incubation, either as a solid particle, or dissolved in 10mM sodium hydroxide as described by Gahmberg *et al.*[4,6]. In order to increase the proportion of sodium borotritide without increasing too much the total amount of radioactivity, various amounts of sodium borohydride (Merck, D-6100 Darmstadt, West Germany) were mixed in some experiments with sodium borotritide.

The labeling was performed with, respectively, 0.012, 0.027, 5.3, 10.6, 16, 20, 26.34, 30, and 41.23 $\mu$mol of sodium borohydride and tritide per $8 \times 10^9$ erythrocytes; as these proportions of reagent resulted, in some experiments, from a mixture of sodium borotritide with sodium borohydride, the specific activity of the resulting isotope varied, according to the experimental conditions, from 7200 Ci/mol to 10 Ci/mol.

After incubation with the isotope, the cells were washed three times with 0.15M sodium chloride and then dissolved in triplicate in 1.5 mL of a 1:1 (v/v) mixture of 0.5M quaternary ammonium hydroxide in toluene (Packard, F-94533 Rungis, France) and 2-propanol (Carlo Erba, F-92081 Paris-La Défense, France); later 35% hydrogen peroxide (0.5 mL) was added in order to discolor hemoglobin; after

gentle shaking, the vials were kept for 1 h at room temperature, and then 1:9 (v/v) 50mM hydrochloric acid–xylene (Packard) (15 mL) was added.

Each vial was prepared in triplicate, and each count of the radioactivity was performed, on each vial in triplicate also, with a liquid scintillation counter (Kontron MR 300). Preliminary experiments showed that the chemiluminescence due to hemoglobin could be diminished by the use of 35% hydrogen peroxide; the maximum amounts of counts were being obtained when 0.5 mL of 35% hydrogen peroxide was added to $25 \times 10^7$ erythrocytes per vial. An experimental quenching curve was obtained with various amounts of erythrocytes ($5–250 \times 10^7$) mixed with a constant and known level of sodium borotritide. By use of this experimental quenching curve, the number of c.p.m. of each vial was converted into d.p.m., and then into nCi. The number of labeled residues per erythrocyte was further determined by using the specific activity of the isotope for each experiment, and then by substracting the nonspecific labeling of the corresponding cells, treated in a manner similar to that of the labeled cells, but without D-galactose oxidase.

*Labeling of young, old, and middle-aged erythrocyte membranes.* — Young, old, and middle-aged erythrocyte membranes were prepared from labeled erythrocytes with hypotonic tris(hydroxymethyl)aminomethane–ethylenediamine tetraacetate [2-amino-2(hydroxymethyl)-1,3-propanediol–1,2-diaminoethane-*N*,*N*,*N*′,*N*′-tetraacetate] buffer as described by Hamaguchi and Cleve[7]. The protein contents of these membranes were determined according to Lowry *et al.*[8]. The content of D-galactose and 2-acetamido-2-deoxy-D-galactose was determined as previously described[9]. The number of labeled residues in the membrane was determined as just described for erythrocytes, but the results are expressed per mg of membranous proteins.

Furthermore, glycoproteins and glycolipids were extracted from the membranes as described by Hamaguchi and Cleve[7]. The count of the radioactivity of these glycoproteins and glycolipids was performed in triplicate on aliquots (0.2 mL) mixed with xylene (10 mL). After substracting the nonspecifically bound isotope, the results of the radioactivity recovered in the glycoproteinic and glycolipidic material were expressed in percentage of the total, specifically bound isotope of the membrane. The radioactivity of the total, protein-bound carbohydrate was that of the membranes minus that of the chloroform–methanol extractable carbohydrate.

## RESULTS AND DISCUSSION

The number of D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues at the cell surface was quantitatively determined by measuring the amount of sodium borotritide necessary to reduce all the sites oxidized by D-galactose oxidase on young, old, and middle-aged $A^+$ red-blood cells from one healthy donor. The number of labeled surface residues per erythrocyte increased with the amount of sodium borotritide added in the incubation medium, until a constant level was reached (Table I and Fig. 1). This constant level, representing the maximal number of sites being

TABLE I

CELL SURFACE LABELING, BY D-GALACTOSE OXIDASE AND BOROTRITIDE, OF YOUNG, MIDDLE-AGED, AND OLD HUMAN $A^-$ ERYTHROCYTES FROM ONE HEALTHY DONOR[a]

| *Nmol of sodium borotritide per 8 × $10^9$ erythrocytes* | *Gal and GalN residues ( × $10^{-6}$) labeled per* | | | | *p of the difference (young cells minus old cells)* |
|---|---|---|---|---|---|
| | *Young cell* | *Middle-aged cell* | *Old cell* | | |
| 12 | 0.085 ±0.013 | 0.040 ±0.006 | 0.021 ±0.003 | | <0.01 |
| 20 | 0.131 ±0.014 | | 0.058 ±0.006 | | <0.01 |
| 27 | 0.266 ±0.022 | 0.10 ±0.009 | 0.096 ±0.009 | | <0.01 |
| 5300 | 16.36 ±2.58 | 7 ±1.36 | 4.48 ±1.26 | | <0.01 |
| 10600 | 35 ±2.60 | 7.6 ±1.49 | 6.87 ±2.38 | | <0.001 |
| 16000 | 40 ±3.10 | 12 ±1.6 | 10 ±1.24 | | <0.01 |
| 20000 | 40 ±4 | 16 ±3 | 12 ±2 | | <0.01 |
| 26300 | 38.37 ±5 | 22 ±4.47 | 17.18 ±3.22 | | |
| 30000 | 40 ±7 | 22 ±4.97 | 18 ±2.6 | | <0.01 |
| 41300 | 38 ±3.05 | 22 ±0.84 | 16 ±3.60 | | <0.01 |

[a]Each experiment was performed in triplicate. Data are expressed as mean ± S.E.

reduced by sodium borotritide, was reached for the young cells with proportions (16 μmol per 8 × $10^9$ cells) smaller than those needed to reach the corresponding constant level for the middle-aged and old cells (26 μmol per 8 × $10^9$ cells) (Fig. 1). Thus, as the amount of D-galactose oxidase of the incubating medium was kept constant, the present results suggest that the accessibility of the oxidized residues to

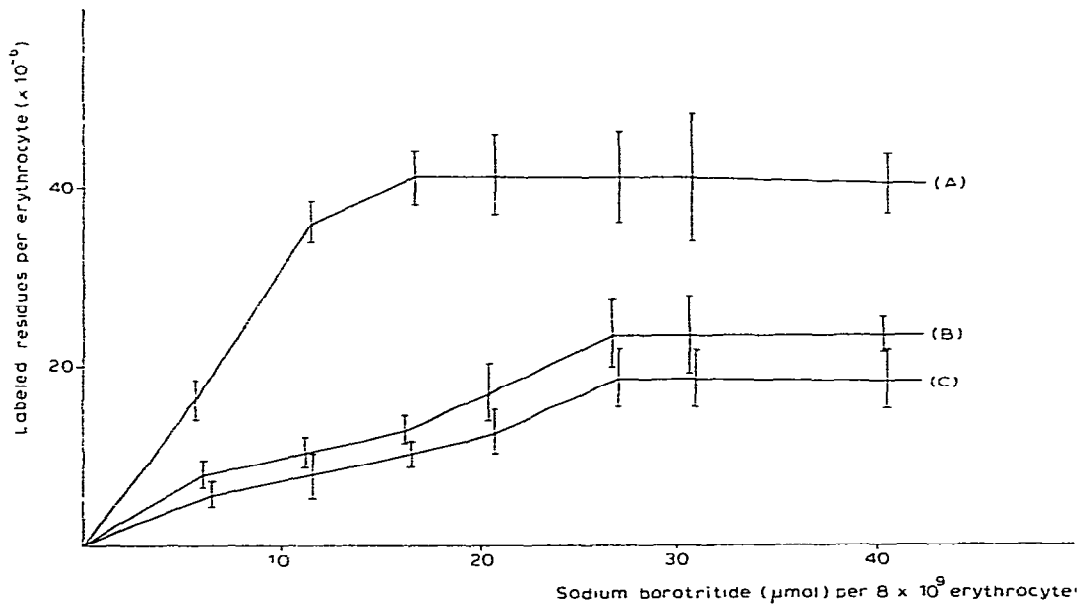


Fig. 1. Cell-surface labeling, by D-galactose oxidase and sodium borotritide, of young (A), middle-aged (B), and old (C) $A^+$ erythrocytes from one healthy, human donor.

sodium borotritide is decreased in the old cells by comparison with the young ones. When the concentration of sodium borotritide was increased in the incubation medium, the number of labeled residues increased much more for the young erythrocytes than for the middle-aged and old ones. The greatest number of sites per $A^+$ erythrocytes that could be labeled was $40 \pm 7 \cdot 10^6$ ($n = 3$) for the young cells, $22 \pm 4.9 \cdot 10^6$ ($n = 3$) for the middle-aged cells, and $18 \pm 2.6 \cdot 10^6$ ($n = 3$) for the old cells ($p$ of the difference between young and old, $<0.01$; $t$, 11.64). Furthermore, for each concentration of sodium borotritide used in the incubation medium, a significant difference occurred between the number of residues labeled for young and old erythrocyte surfaces (Table I). Thus, in addition to the decrease of accessibility to sodium borotritide, it was observed that the maximal number of residues per erythrocyte of old cell that can be labeled under the conditions of complete reduction was decreased. These experiments showed that a complete reduction of the D-galactose-oxidized residues of young, old, and middle-aged $A^+$ erythrocytes occurred when 27 $\mu$mol of sodium borotritide was added to the incubation medium for $8 \times 10^9$ erythrocytes. The experimental conditions just described, which give a complete reduction of the surface D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues oxidized by D-galactose oxidase, were applied to determine the total number of residues at the surface of young, old, and middle-aged erythrocytes from eight $A^+$ healthy donors. Under these experimental conditions, the nonspecific labeling of the young erythrocytes was $1.69 \pm 0.85\%$ ($n = 16$), that of the old $2.16 \pm 0.72\%$ ($n = 16$), and that of the middle-aged $2 \pm 0.80\%$ ($n = 16$). No significant difference occurred between the nonspecific labeling of the young and of the old cells ($t$ of the difference between young and old 0.9; $p$ non-significant).

Furthermore, the specific labeling was $3.11 \pm 0.86\%$ ($n = 16$) for the young cells and $1.76 \pm 0.68\%$ for the old cells ($n = 16$). Thus, a significant difference was observed between the specific labeling of the young and old erythrocytes ($t$ 2.38; $p < 0.05$). The average number of labeled residues per one young erythrocyte was $37.6 \pm 1.8 \times 10^6$, per one middle-aged $24.8 \pm 6.4 \times 10^6$, and per one old $21.8 \pm 4.9 \times 10^6$. Thus, a significant decrease between the number of labeled residues at the surface of young and old $A^+$ erythrocytes was observed ($t$ 7.25; $p < 0.001$; $n = 8$). The membranes from young, old, and middle-aged erythrocytes labeled with a saturation dosis of sodium borotritide contain $400 \pm 50 \times 10^{14}$ ($n = 4$) of labeled residues per mg of protein for the young erythrocyte membrane, $224 \pm 57 \times 10^{14}$ ($n = 4$) for the old, and $293 \pm 10 \times 10^{14}$ for the middle-aged. The total residues of the young and old erythrocyte membrane was also determined (Table II).

Thus, a significant decrease of the surface D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues occurs during human erythrocyte *in vivo* ageing. Whereas the decrease of the surface area of the old red blood cells during ageing is[9] $\sim 10$–$11\%$, that of the total membranous D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues is 12.5% (Table II), and that of these residues at the cell surface is 42%. These data suggest that rearrangements of the erythrocyte membrane occur during ageing. Furthermore, the protein-bound carbohydrate of the young erythrocyte

TABLE II

TOTAL AND SURFACE GAL AND GALN RESIDUES CONTENT OF YOUNG AND OLD $A^+$ ERYTHROCYTES MEMBRANES

| *Residues* | *Number of residues ($\times 10^{-11}$) in*[a] | | *p of the difference (young cells minus old cells)* |
|---|---|---|---|
| | *Young-erythrocyte membrane* | *Old-erythrocyte membrane* | |
| Total Gal | 1037 ±126 | 907 ±89.52 | <0.01 |
| Total GalN | 410 ±32.4 | 358 ±116.4 | <0.01 |
| Total Gal and GalN | 1447 | 1264 | |
| Labeled Gal and GalN | 400 ±50 | 224 ±57 | <0.001 |
| Labeled/total Gal and GalN | 0.276 | 0.180 | |

[a]Results are expressed in residues per mg of membranous protein, and as mean ± S.E.

membrane contains an average of 60 ±6.6% of the surface-labeled residues, whereas those of the old erythrocyte membrane is only an average of 46 ±6.2% ($p$ of the coupled difference between young and old $<0.05$; $n = 5$; $t$ 3.6). Thus, a rearrangement in the organization of these surface residues between glycoproteins and glycolipids occurs during ageing. The results reported in this study show that the analysis of the whole membrane does not reflect the changes occurring at the surface of the cells, and that D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues are masked in the old cell-surface.

Recently Choy *et al.*[10] have shown, for old cells, an increase of some D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues by the use of *Ricinus communis II* and *Bauhinia purpurea* agglutinins. The number of labeled residues that these authors observed represent <10% of the total surface residues labeled by D-galactose oxidase and sodium borotritide in the conditions of complete reduction of the oxidized residues. Thus, in addition to the 42% decrease of the surface residues reported earlier, a small increase of certain residues may occur during ageing. The discrepancy between the results of Choy *et al.*[10] and ours may be explained by a difference in specificity between enzymes and lectins.

The masking of the surface D-galactosyl and 2-acetamido-2-deoxy-D-galactosyl residues observed in the present study may be due to spatial rearrangements of the membrane components. Indeed, Kadlubowski[11] and ourselves[12] have observed, by polyacrylamide gel-electrophoresis of young and old human-erythrocyte ghosts, changes in the percentage of some of the protein components stained by Coomassie Blue. Masking may be due either to the binding of IgG immunoglobulins on new antigenic sites of the old-erythrocyte surface-membrane[13], or to a deeper rearrangement of the surface carbohydrate component of the membrane during erythrocyte ageing.

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