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in vivo AGEING OF HUMAN ERYTHROCYTES AND CELL-SURFACE LABELING BY METAPERIODATE AND SODIUM BOROTRITIDE

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ABSTRACT

Young and old human erythrocytes, separated *in vitro* according to age, were labeled at the surface-sialic acid residues by sodium periodate and borotritide treatment. No qualitative difference was observed between the sialic acid derivatives of young- and old-erythrocyte membranes. The number of labeled residues was significantly decreased in the *in vivo* aged erythrocytes ($12 \pm 2.8 \times 10^6$; n = 8) when compared to the young ones ($19.4 \pm 2.8 \times 10^6$; n = 8). Analysis of the labeled glycoproteins by sodium dodecyl sulfate gel-electrophoresis showed that this decrease affects the PAS-1, PAS-4, PAS-2, and PAS-3 bands (glycophorins A and B) of the old-erythrocyte membranes.

INTRODUCTION

We have previously shown[1–3] that the significant decrease of the sialic acid residues occurring during *in vivo* ageing of the human erythrocytes affects glycophorin A. The present study was undertaken in order to elucidate (a) whether glycophorin B is also involved in the decrease of the surface-sialic acid residues of the old human erythrocytes, and (b) whether the sialic acid derivatives of the young- and old-erythrocyte membranes are similar or qualitatively different. For these purposes, young and old human erythrocytes were separated *in vitro* according to their age *in vivo*, and the cell-surface sialic acid residues labeled by 2mM periodate oxidation and borotritide reduction. The number of labeled residues per young and old erythrocytes was quantitatively determined, the sialic acid derivatives were identified by paper chromatography[4], and the compounds containing the sialic acid residues in the erythrocyte membranes were analyzed by sodium dodecyl sulfate gel-electrophoresis.

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EXPERIMENTAL

Separation of erythrocytes of different ages. — Blood samples, freshly drawn from normal donors of different ABO groups, were collected into heparinized tubes and treated separately, immediately after collection. As the density of erythrocytes increases with ageing[5], the erythrocytes of different ages were separated by centrifugation for 2 h at 2000*g* at 18°, as previously described[5]. The white blood cells were removed from the young, red blood cells by aspiration. Thus, the remaining white blood-cells include $< 0.2\%$ of the total cell count and the remaining platelets $< 0.5\%$ of the total cell count. The differences in age of the erythrocytes were assessed by comparing the pyruvate kinase (EC 2.7.1.4.0) and the cholinesterase (EC 3.1.1.7.) activities as reported earlier[1].

Sodium periodate–borotritide treatment of young, old, and middle-aged erythrocytes. — The introduction of tritium label into sialoglycoproteins, present within the intact erythrocytes, was performed as described by Gahmberg and Andersson[6] with slight modifications in the use of different proportions 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 periodate-oxidized residues.

The washed erythrocytes ($8–9 \times 10^9$ cells/mL) were incubated for 10 min in the dark, at + 4°, with equal volumes of 2mM sodium periodate (Merck) and 0.2mM phenylmethanesulfonyl fluoride (Sigma) dissolved in phosphate buffer saline, pH 7.4 (PBS). 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 sodium periodate. 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 (Dreieichenhain, West Germany) or from the Radiochemical Centre (Amersham, France). The sodium borotritide was added to PBS just before incubation, dissolved in 10mM sodium hydroxide as described by Gahmberg *et al.*[7]. In order to increase the proportion of sodium borotritide without increasing too much the total amount of radioactivity, various amounts of sodium borohydride (Merck) were mixed in some experiments with sodium borotritide. The specific activity of the resulting isotope varied, according to the experimental conditions, from 10 000 to 50 Ci/mol.

After incubation with the isotope, the cells were washed three times with 0.15M sodium chloride, and then dissolved in triplicate in 1:1 (v/v) 0.5M quaternary ammonium hydroxide in toluene (Packard)–2-propanol (1.5 mL); 35% hydrogen peroxide (0.5 mL) was added subsequently to decolor hemoglobin. After being gently shaken, 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, with a liquid-scintillation counter (Kontron MR 300). Preliminary experiments showed that the chemiluminescence due to hemoglobin could be di-

minished by treatment with 35% hydrogen peroxide; the maximum amounts of counts were obtained when 0.5 mL of 35% hydrogen peroxide was added to 25×10^7 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. 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 determined by use of 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 periodate.

Identification of tritiated sialic acid derivatives. — In some experiments, erythrocytes were labeled, as just described, with sodium borotritide of high-specific activity (10 000 Ci/mol). The labeling was performed with 1.6 mCi of the isotope for 8×10^9 cells. The complete reduction of the periodate-oxidized sialic acid residues was achieved with sodium borohydride (Merck, 1 mg dissolved in P.B.S.) for 8×10^9 cells. The labeled erythrocytes were hydrolyzed for 1 h at 80° in 50mM sulfuric acid. The hydrolyzates were centrifuged at 3000 r.p.m. for 10 min, the pellets removed, and the pH of the acid supernatant solutions was adjusted to 6. These solutions were counted for radioactivity.

^{3}H-Labeled C-7 and C-8 *N*-acetylneuraminic acids were prepared as previously described[4]. The labeled products were chromatographed on Whatman No. 3 paper with 5:5:3:1 (v/v) pyridine–ethyl acetate–acetic acid–water. The paper strips containing the radioactive samples were cut into 1-cm pieces and placed into scintillation vials and the radioactivity was determined. Control samples were treated identically, except for the addition of periodate in order to determine the specific radioactivity (total minus nonspecific radioactivity).

Young, old, and middle-aged erythrocyte membranes. — Membranes were prepared from labeled erythrocytes with hypotonic Tris–ethylenediaminetetraacetate buffer as described by Hamaguchi and Cleve[8]. The protein contents of these membranes were determined according to Lowry *et al.*[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. In some experiments, glycophorin A was isolated by affinity chromatography as described by Kahane *et al.*[10].

Sodium dodecyl sulfate gel-electrophoresis. — Membranes from periodate–borotritide erythrocytes and from borotritide-treated erythrocytes (without periodate) were analyzed on parallel gels by polyacrylamide gel-electrophoresis in sodium dodecyl sulfate in Tris buffer (pH 7.4) as described by Fairbanks *et al.*[11], but slabs containing a gradient of 4 to 30% polyacrylamide gel were used instead of uniform, cylindrical polyacrylamide gels. The glycoproteins from the erythrocytes treated with borotritide (without periodate) were then stained for carbohydrates as described by Glossmann and Neville[12]. The proteins from each electrophoresis experiment were stained with Coomassie Blue as described by Fairbanks *et al.*[11]. The gels were calibrated with molecular-weight markers, phosphorylase B (M_r 94 000),

bovine serum albumin (M_r 67 000), and soybean trypsin inhibitor (M_r 21 500) (Pharmacia).

Prior to electrophoresis, the membranes (2 vol.) were solubilized in 0.15M Tris buffer (pH 6.7) containing 3% of $NaDodSO_4$, 30% of glycerol, and 4.5% of β-mercaptoethanol (1 vol), and treated for 30 min at 37°. An amount of 40 μg of membrane proteins was applied in each electrophoresis experiment. In some experiments, the hemolyzates from the labeled erythrocytes were solubilized as just described, and submitted to polyacrylamide gel-electrophoresis. After electrophoresis of the membrane and staining with Coomasie Blue or the periodic acid–Schiff reagent (PAS), the gels were scanned at 560 nm on a DCD 16 Gelman instrument.

Four coupled experiments were performed with young, middle-aged, and old labeled erythrocyte membranes and their hemolyzates prepared from four different donors. In each experiment, the mobilities of the radioactive peaks were compared to those of the corresponding intact sialoglycoproteins from the whole membranes stained as described by Glossman and Neville[12], and to those of the isolated glycophorin A. Radioactivity was determined in the Coomasie Blue-stained gels; for the counting, 2-mm gel slices were cut promptly, because the modified sialic acid that contains the radioactive label is labile in acetic acid, and they were immediately placed in Soluene 100 (1 mL, Packard) for 36 h; then a scintillation cocktail (10 mL) was added. C.p.m. were measured with a Kontron MR 300 liquid-scintillation counter. The glycoproteins were designated as PAS-1, PAS-2, PAS-3, and PAS-4.

RESULTS AND DISCUSSION

When intact erythrocytes were subjected to sequential mild-oxidation and reduction with sodium borotritide, the tritium label predominantly appeared in the sialoglycoproteins (Fig. 1). A large proportion (73 ± 10%) of the specific radioactivity was released from the cells by mild acid hydrolysis, almost all being recovered as C-7 and C-8-neuraminic acid (Fig. 2). The ratio of C-7- to C-8-neuraminic acid was the same for the material prepared from the young red-blood cells and for that prepared from the *in vivo* aged ones (Fig. 2), indicating no preferential substitution of OH-7 and -8 during *in vivo* ageing.

The number of labeled residues at the cell surface was quantitatively determined by measuring the amount of sodium borotritide necessary to reduce all the sites, oxidized by 2mM periodate, on young, old, and middle-aged red-blood cells from one healthy donor (Fig. 3). The number of labeled cell-surface residues per erythrocyte increased with the amount of sodium borotritide added to the incubation medium, until a maximum level was reached (Fig. 3). This maximum level represents the maximal number of sites being reduced by sodium borotritide. Furthermore, for each concentration of sodium borotritide used in the incubation medium, a significant difference occurred between the number of residues labeled for young-

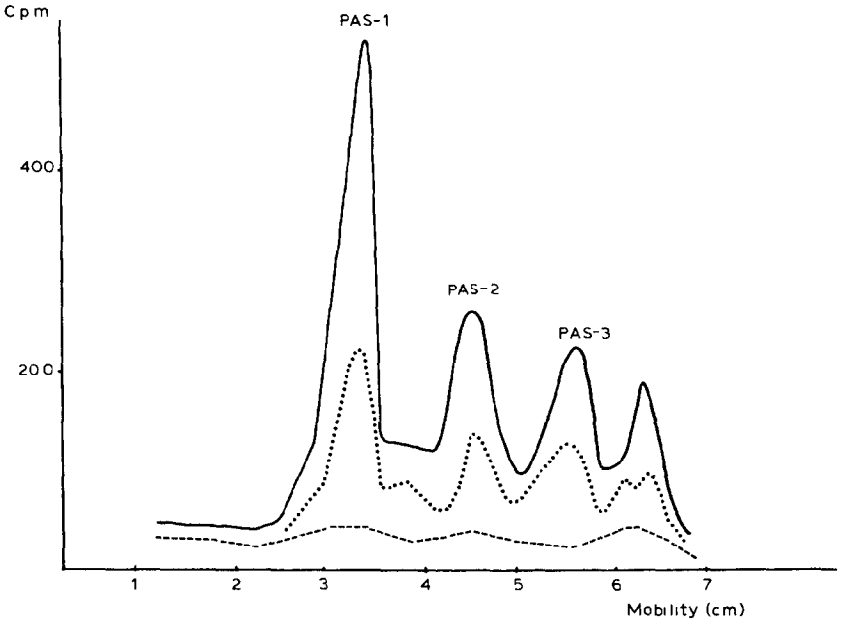

Fig. 1. Polyacrylamide gel-electrophoresis of membranes treated with periodate and sodium borotritide: (——) young-erythrocyte membranes, (· · · · ·) old-erythrocyte membranes, (----) membranes treated with sodium borotritide only.

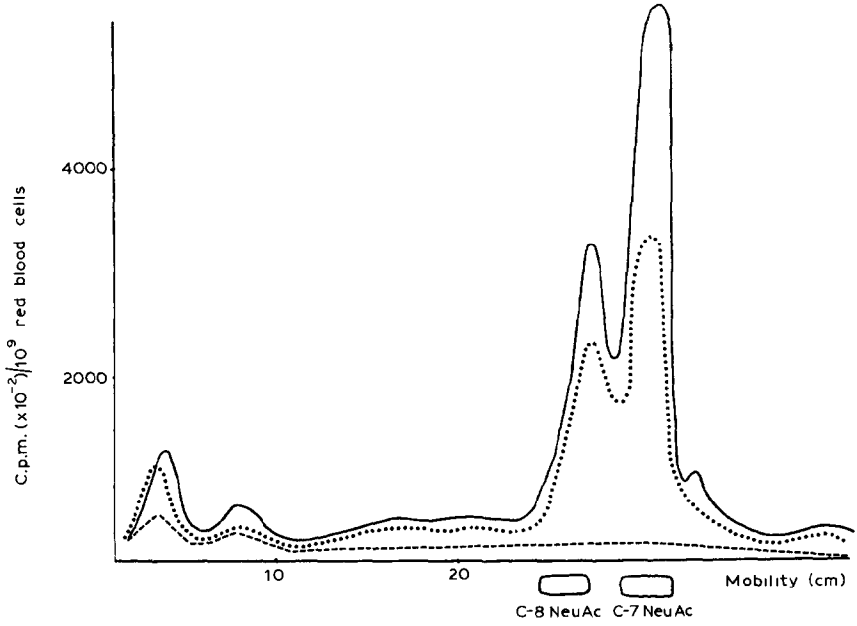

Fig. 2. Chromatography in 5:5:3:1 (v/v) pyridine–ethyl acetate–acetic acid–water, of sulfuric acid hydrolyzates from young and old erythrocytes: (——) young erythrocytes, (· · · · ·) old erythrocytes treated with metaperiodate and sodium borotritide, and (----) erythrocytes treated with borotritide only.

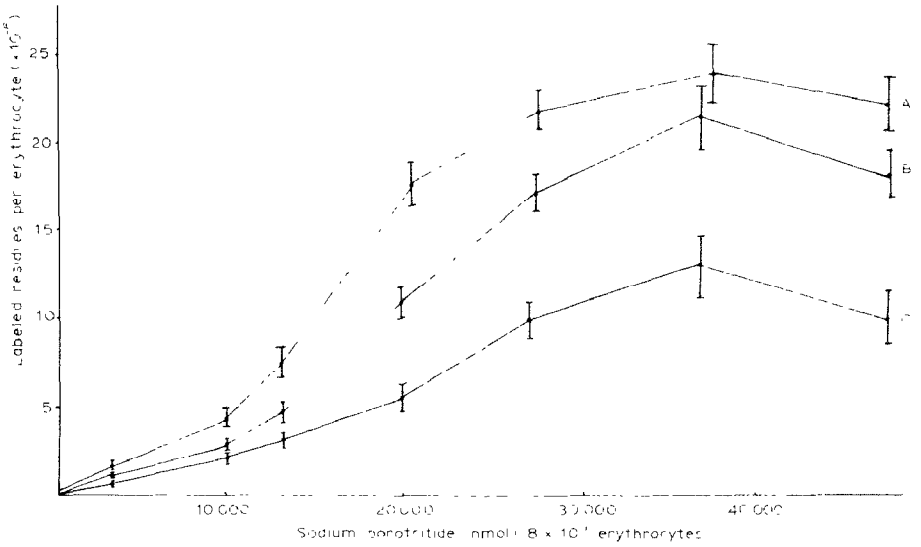

Fig. 3. Cell-surface labeling by periodate and sodium borotritide of: (A) young, (B) middle-aged, and (C) old human erythrocytes from one healthy human donor.

and old-erythrocyte surfaces (Fig. 3). In some experiments, membranes from labeled erythrocytes were prepared. In each case the number of labeled residues, expressed per mg of membraneous protein, was significantly decreased on the old cell-surface membranes (Table I).

The experimental conditions just described, which give a complete reduction of the surface residues oxidized by 2mM periodate, were applied to determine the total number of labeled residues at the surface of young, old, and middle-aged-erythrocytes from eight healthy donors. The average number of labeled residues was $19.4 \pm 2.8 \times 10^6$/young, $16.2 \pm 2.9 \times 10^6$/middle-aged, and $12 \pm 2.6 \times 10^6$/old cell. Thus, a significant decrease (38%) of labeled residues at the surface of young and old erythrocytes was observed ($p < 0.001$); it is slightly higher than that (22%) reported for the surface sialic acid residues liberated by neuraminidase treatment[1] Thus, these results suggest a possible steric hindrance in old cells, to periodate oxidation–borotritide reduction.

Four coupled experiments of electrophoresis of membranes and of hemolyzates, obtained from young and old erythrocytes labeled on their cell surface by periodate–borotritide, were performed. The electrophoretic pattern of the radioactive peaks was compared, in each case, to that of the intact sialoglycoproteins stained by PAS and to that of the isolated glycophorin A. The mobilities of the radioactive peaks correspond, in each case, to the mobilities of PAS-1, PAS-4, PAS-2, and PAS-3 glycoproteins stained by PAS. The mobilities of the various radioactive peaks were similar for the young and for the old erythrocyte-membrane glycoproteins. However, in each case a decrease in the labeling of the peaks corresponding to PAS-1, PAS-4, PAS-2, and PAS-3 glycoproteins from the old-erythro-

TABLE I

CELL-SURFACE LABELING BY PERIODATE AND BOROTRITIDE OF YOUNG, MIDDLE-AGED, AND OLD HUMAN ERYTHROCYTES[a]

Sodium borotritide (nmol/ 8×10^9 erythrocytes)	*Labeled residues ($\times 10^{-10}$)/mg of membraneous protein*			*P of the difference (young less old cells)*
	Young cells	*Middle-aged cells*	*Old cells*	
80	255 ± 21		91 ± 10	< 0.01
300	5200 ± 250	2800 ± 180	2050 ± 200	< 0.001
1600	112000 ± 500	55200 ± 350	48000 ± 200	< 0.001

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

cyte membranes was observed, in comparison with the specific labeling observed for the corresponding peaks from the young-erythrocyte membranes (Fig. 1). In agreement with previous reports[13,14], the isolated glycophorin A migrates in the positions corresponding to PAS-1, PAS-4, and PAS-2. In addition, Furthmayr and assoc.[10,15,16] have reported that PAS-3 corresponds to glycophorin B. Aggregates between glycophorin A and B and between glycophorin B molecules have been described[17]. Such aggregates, which occur in a concentration-dependent fashion and especially in isolated glycoproteins, migrate[17] in the gel in a region between PAS-1 and PAS-2.

If such aggregates occur in the present work, PAS-1 and PAS-2 would nevertheless represent glycophorin A and PAS-3 glycophorin B. In addition, the same amount of membrane proteins was applied in each electrophoresis experiment, and a decrease of the labeling of the glycoproteins of the old-, as compared to the young-erythrocyte membrane, was observed in the region of the gel between PAS-1 and PAS-2. Thus, it may be concluded that the decrease of the cell-surface sialic acid residues, labeled on the intact cells by sequential periodate and borotritide treatment, affects both glycophorin A and glycophorin B.

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