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CONTENT OF RED BLOOD-CELL SIALIC ACID IN BW 35 BLOOD DONORS. RELATION TO MAGNESIUM CONCENTRATION AND PYRUVATE KINASE ACTIVITY*

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ABSTRACT

Previous studies have shown that the concentration of red blood cell (RBC) magnesium is significantly lower in subjects carrying an HLA-BW 35 antigen ($p < 0.001$) than in non-carriers. As this finding might be related to modifications of the RBC membrane sialoglycoconjugates, RBC sialic acid was comparatively determined in BW 35^+ and BW 35^- subjects. Pyruvate-kinase activity, mean RBC volume, and reticulocyte count have also been determined in order to estimate whether some significant variations in the level of these age markers could be detected between the HLA BW 35^+ and BW 35^- subjects. A significant negative correlation between sialic acid and RBC magnesium concentrations was observed for the whole population tested (n 57, $p < 0.005$), 61% of the BW 35^+ and only 25% of the BW 35^- individuals having sialic acid values above, and magnesium values below the overall mean ($p < 0.01$). The variance of mean RBC volume was also larger for the BW 35^+ group. Other determinations did not show any significant variations, suggesting that the results are not related to RBC age.

INTRODUCTION

Previous studies performed on 351 healthy blood donors have shown that the concentration of red blood cell (RBC) magnesium is significantly lower in subjects carrying an HLA BW 35 antigen ($p < 0.001$) than in non-carriers[1–4]. In order to detect, in a first approach, whether this finding might be related to modifications of

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the RBC membrane sialoglycoconjugates, we studied, in the same groups of subjects, the RBC membrane sialic acid.

As the magnesium concentration in RBC *in vivo* varies with the age of the cell, namely very high in reticulocytes[5], reaching a steady state[6] in mature RBC, and then decreasing again slightly[7], we envisaged the hypothesis that the lower magnesium content of the RBC from the HLA BW 35^+ blood donors might be related to the older, mean age of the cells. To test this hypothesis, we studied comparatively, in the two groups of subjects, the values of some RBC constituents that are known to vary significantly, with the age of the cell, such as the pyruvate kinase activity[8] (PK), RBC volume (MCV), and reticulocyte count, trying to establish whether the magnesium content of the RBC might be correlated to some of these parameters. As the sialic acid of the RBC decreases significantly as the cell ages *in vivo*[9–13], the correlation between sialic acid and magnesium contents was also investigated.

EXPERIMENTAL

In order to avoid seasonal variations, the experiments were conducted from October to February on 57 healthy, regular blood donors (unrelated adult males) of known HLA groups, coming from the panel of the INSERM unit U 93 (Professor Jean Dausset) and the Transfusion Center of Hôpital Saint Louis (Professor J. Reviron). Blood (10 mL), taken in the morning before breakfast, in heparinized tubes was immediately centrifuged at 1000*g* for 10 min in order to separate plasma and red cells. For Mg determination, a second centrifugation of red cell was carried out and the buffy coat removed to eliminate leucocytes and platelets. The Mg content was determined in duplicate on red cell hemolyzates by atomic absorption spectrophotometry as recommended by Rousselet[14] and expressed in mg/L of RBC. The reproductibility of the method has been estimated[3,4] to be 1.3%.

For sialic acid determination, the red cells were first washed three times with 0.15M NaCl. The buffy coat was removed and, after each centrifugation, leucocytes and platelets were removed by aspiration. The final RBC suspension contained less than 0.1% of white cells and less than 0.15% of platelets. The RBC suspension (1 vol.) was then incubated for 90 min at 37° with *Vibrio cholerae* neuraminidase (VCN) (Behringwerke, West Germany), 60 U/8 · 10^9 erythrocytes in phosphate buffer saline (2 vol.), pH 7.4, as previously described[15]. In each experiment, VCN (60 U) dissolved in 50mM sodium acetate, 0.154M NaCl, 9mM $CaCl_2$, pH 5.5, buffer (60 μL) was diluted by phosphate buffer saline (PBS) (2 mL). In some experiments, parallel incubations with the upper neuraminidase solution were performed in PBS containing 10mM $MgCl_2$ or in 0.15M NaCl containing 10mM $CaCl_2$. After incubation, the cells were centrifuged off for 10 min at 1000*g*, and free sialic acid was determined in the supernatant solution by the thiobarbituric reaction, as described by Warren[16]. An aliquot of the RBC suspension was counted in triplicate in an electron Coulter. Another aliquot was used for hematocrit determination, the

mean corpuscular volume (MCV) being calculated from these data. The results are expressed in μg of sialic acid per 10^{10} RBC. The variability coefficient calculated from 5 determinations on the same blood sample is 4%.

In some experiments, membranes from intact and desialylated RBC were prepared by hemolysis of RBC at +4° with hypotonic 5mM phosphate buffer, pH 7.4 (30 vol.). The hemoglobin-containing ghosts were centrifuged off at 17 000*g* for 20 min at +4°. White membranes were obtained after three washings with the buffer. Total bound sialic acid was released by acid hydrolysis as follows. Membranes (1 vol.) were incubated with 50mM H_2SO_4 (2 vol.) for 1 h at 80°. Free sialic acid was determined as earlier described and expressed per mg of membrane protein. Proteins were measured according to Lowry *et al.*[17].

The pyruvate kinase (PK) activities, as described by Beisenherz[18] and expressed in U.I. per 10^{10} RBC, and the reticulocytes counts after Cresyl Blue coloration were determined on a reduced number of subjects. In some experiments, erythrocytes were separated[19], *in vitro* according to their age, by differential centrifugation for 2 h at 2000*g* and at 18° using centrifuge tubes of 0.5-cm bore and 8-cm length as previously described[20]. The top and bottom 5% of the fractions, enriched in younger and older cells, respectively, were used for sialic acid and PK determinations as described earlier.

The significance of difference between means was estimated by the Student test. The variance and distributions of the values of the two groups studied were respectively compared by calculating F ratios and X_2 values. The tests of significance of difference between correlations and the average correlation coefficients were computed from the group correlation coefficients, after Fisher Z transformation. Covariance analysis was used for the comparison of regression lines.

RESULTS AND DISCUSSION

The quantities of sialic acid released from red cells treated for 90 min at 37° with VCN at a concentration of 75 units per 10^{10} RBC represent 94 ±3.5% (n 10) of the total sialic acid of the RBC, as assessed by the ratio "sialic acid per mg of membranous proteins from neuraminidase-treated RBC/sialic acid per mg of membranous proteins from intact RBC". Parallel incubation of RBC with the neuraminidase solution diluted in PBS, or in PBS containing 10mM magnesium chloride, or in 0.15M sodium chloride containing 10mM calcium chloride gave statistically similar results (n 7). These results of desialylation are in close agreement with those obtained by other investigators[21,22]. It is known that in human RBC, 95–100% of sialic acid is in the form of *N*-acetylneuraminic acid[21,23] removable by VCN.

In the present study, in order to be able to compare the sialic acid contents, per red blood cell, of the HLA BW 35^+ and BW 35^- blood donors, the sialic acid removable by VCN was determined. The level of this sialic acid is significantly higher in the RBC of BW 35^+ than in that of BW 35^- blood donors ($p < 0.05$)

TABLE I

MEANS AND STANDARD DEVIATIONS OF THE VARIABLES DETERMINED IN TWO GROUPS OF BLOOD DONORS, CLASSIFIED FOLLOWING THE PRESENCE OR ABSENCE OF THE BW 35 ANTIGEN

Variables	*BW 35⁺*			*BW 35⁻*			*Total*		
	n	*m*	*σ*	*n*	*m*	*σ*	*n*	*m*	*σ*
Sialic acid ($\mu g/10^{10}$ RBC)	18	186.98[a]	16.76	39	175.44[a]	18.41	57	179.08	18.56
RBC Mg (mg/L)	18	51.10	4.08	39	53.08	4.32	57	52.45	4.31
Pyruvate kinase ($U/10^{10}$ RBC)	11	1.923	0.625	24	2.145	0.551	35	2.075	0.575
RBC mean corpuscular vol. (μm^3)	18	86.57	7.22[b]	39	89.16	4.63[b]	57	88.34	5.64
Reticulocytes (%)	10	0.51	0.19[a]	19	0.62	0.30[a]	29	0.58	0.27
Age (years)	18	44.9	14.7	39	45.5	14.0	57	45.3	14.1

[a]Means or variances different at the level of $p < 0.05$ or [b]$p < 0.01$.

TABLE II

DISTRIBUTION OF BW 35⁺ AND BW 35⁻ SUBJECTS ACCORDING TO RBC *N*-ACETYLNEURAMINIC ACID AND MAGNESIUM CONTENT[a]

RBC Magnesium (mg)	*RBC N-acetylneuraminic acid (μg)*	
	Low (≤179)	*High (>179)*
High (>52.5)	4 BW 35⁺ (22%) 16 BW 35⁻ (41%)	2 BW 35⁺ (11%) 7 BW 35⁻ (18%)
Low (≤51.5)	1 BW 35⁺ (6%) 6 BW 35⁻ (15%)	11 BW 35⁻ (61%) 10 BW 35⁻ (26%)

[a]High and low are defined by reference to the overall means (see Table I). The ratio of BW 35⁺ to BW 35⁻ subjects in the lower right quadrant (high NeuAc, low Mg) is significantly different (χ^2 6.7, $p < 0.01$) from the proportion observed in the three other sections.

TABLE III

CORRELATION COEFFICIENTS[a] (r) CALCULATED ON THE TWO GROUPS OF SUBJECTS AND ON THE TOTAL POPULATION STUDIED

Groups	*RBC NeuAc and Mg content*			*RBC NeuAc content and PK activity*		
	df	*r*	*p*	*df*	*r*	*p*
BW 35⁺	16	−0.621	<0.01	9	+0.562	n.s.
BW 35⁻	37	−0.273	n.s.	22	+0.552	<0.01
Total	55	−0.408	<0 005	33	+0.417	<0.025
Average r	54	−0.390	<0.005	32	+0.555	<0.001

[a]Abbreviations: correlation coefficient, r; degree of freedom, df; threshold of significance, p; and non-significant, n.s. The average value was calculated for the group correlation coefficients after Fisher Z transformation.

(Table I). Furthermore, the difference between the RBC of the BW 35^+ and BW 35^- subjects appears clearly significant when the individuals were classified according to both sialic acid and magnesium content ($p < 0.01$) (Table II). Most of the BW 35^+ values (11 subjects, *i.e.*, 61%) are characterized by sialic acid content values higher than the mean, and magnesium content values lower than the mean ($p < 0.01$) (Table II). These results can also be expressed as sialic acid-to-magnesium ratios. These ratios are normally distributed and their means are 3.73 ±0.11 (s.e.m.) for the BW 35^+ groups and 3.33 ±0.08 for the BW 35^- groups (s.e.m.) ($p < 0.001$). The magnesium content values of this study closely fits previous results[1–4]. But, as the number of subjects presented herein is significantly smaller (57 instead of 351), the difference between the magnesium content values of the BW 35^+ and the BW 35^- group does not reach, here, the level of statistical difference observed for the whole population previously tested[1–4] (Table I).

A significant, negative correlation was observed between the sialic acid content of the RBC and the magnesium concentration of the population tested ($p < 0.005$) (Table III). This correlation, described herein for the first time, cannot be attributed to variations in mean cell-age since older cell populations have at the same time a lower sialic acid content and a lower magnesium concentration. The present findings rather suggest that cell membranes having a "high" sialic acid content would have properties leading either to a greater loss of magnesium from within the cell or to decreased cell ability to actively concentrate magnesium. Variations in the pre-erythrocytes maturation time may also be involved, as a longer maturation would allow a greater magnesium loss from the cell[6], and the

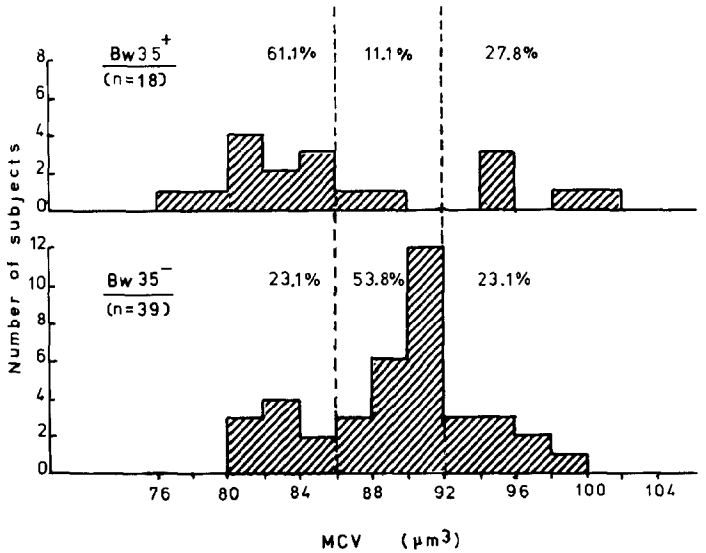

Fig. 1. Distribution of mean corpuscular volumes (MCV) in the two groups of subjects studied. When divided in three class intervals, these distributions are different at the level of $p < 0.005$.

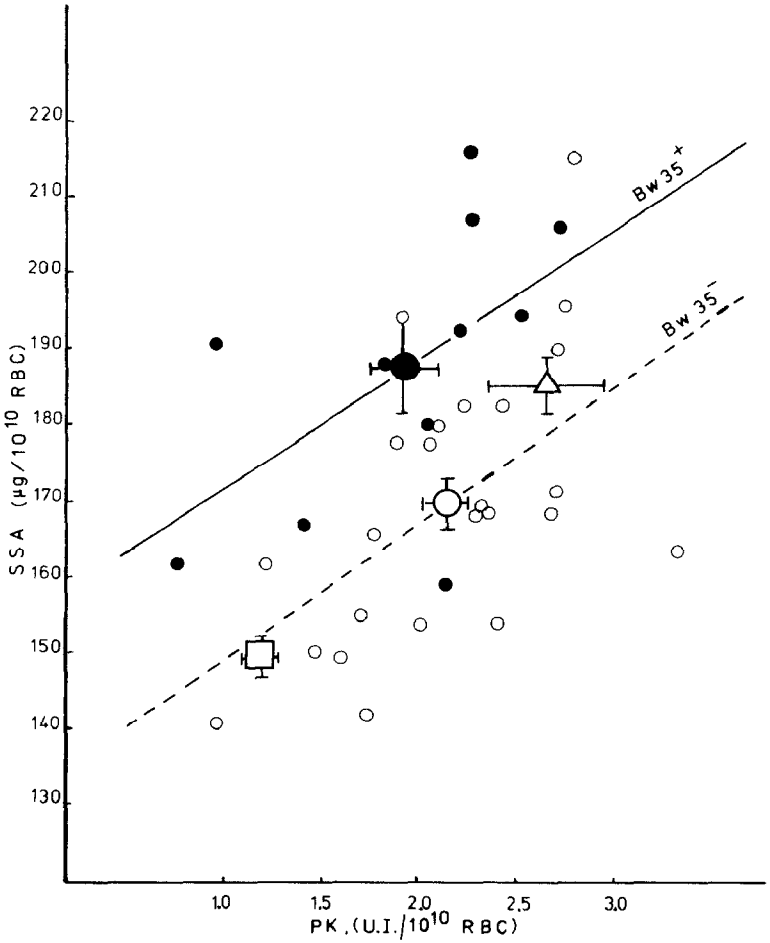

Fig. 2. Relationship between surface sialic acid (SSA) and pyruvate kinase (PK) activity in the two groups studied. Small symbols: individual values of BW 35$^+$ (●) and BW 35$^-$ (○) subjects. Large symbols: means and standard errors of the BW 35$^+$ (●), the BW 35$^-$ group (○), a younger RBC fraction (△) and an older RBC fraction (□). The two regression lines are calculated only on the individual values of BW 35$^+$ and BW 35$^-$ subjects (see text).

synthesis of larger quantities of glycophorin or a more complete sialylation of oligosaccharide chains[24]. The involvement of sialoglycoproteins in RBC-membrane permeability to Mg remains to be investigated. Under pathological conditions, such as leukemia or aplasia, we have observed a significant decrease of RBC sialic acid and a concommitant increase of the magnesium concentration. No significant difference was observed between the means values of the MCV, PK activity, and reticulocyte count of the BW 35$^+$ and of the BW 35$^-$ groups (Table I). This confirms that the significant difference of the ratios of sialic acid to magnesium observed between the RBC from BW 35$^+$ and BW 35$^-$ blood donors are not related with concommitant variations of the mean values of the age indicators tested. How-

ever, a very different distribution of the MCV values among the BW 35$^+$ and the BW 35$^-$ individuals was observed (Fig. 1). Furthermore, the correlation coefficients between sialic acid content and PK activity of the RBC were positive and almost identical for the two groups (Table III); the regression lines are strictly parallel with a translation toward higher sialic acid content values for the BW 35$^+$ group (Fig. 2). The height of the regression line above the x axis (parameter a in $y = bx + a$, where sialic acid = y and PK = x), *i.e.*, the sialic acid content value for a given PK value, is significantly ($p < 0.01$) higher in the BW 35$^+$ group than in the BW 35$^-$ group as shown by covariance analysis. The average r value was also highly significant ($p < 0.001$).

For comparison, younger and older erythrocytes from different blood samples taken from healthy subjects of unknown HLA groups were separated (Fig. 2). As previously reported[10], the differences between younger and old fractions are significant for both sialic acid content ($p < 2 \cdot 10^{-7}$) and PK determinations ($p < 2 \cdot 10^{-4}$). These differences roughly fit the regression line drawn between the values determined for the unseparated erythrocytes (all age fractions mixed) of our BW 35$^-$ group (Fig. 2). This result demonstrates the role played by the RBC mean age in the PK-to-NeuAc correlation *within* the BW 35$^-$ group. In contrast, the difference *between* the two HLA groups follows a slope almost perpendicular to that observed between cell-age fractions, indicating the different origins of these two sources of variations.

Thus, it may be concluded that the significant difference between the sialic acid-to-magnesium ratios of the RBC from BW 35$^+$ and BW 35$^-$ blood donors are not related to significant variations in the mean values of the age indicators tested, *i.e.*, MCV, PK, and reticulocyte count. The physiological meaning of the relationship between sialic acid content, magnesium content, and HLA BW 35 antigen deserves further study.

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