Oxygen-Binding Characteristics of Erythrocyte in Children with Type I Diabetes Mellitus of Different Duration

V. A. Mityanina, E. Yu. Parshina, A. I. Yusipovich, G. V. Maksimov, and A. A. Selischeva

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 4, pp. 499-503, April, 2012
Original article submitted February 1, 2011

Oxygen-binding properties of erythrocyte hemoglobin were studied in children with type 1 diabetes mellitus by Raman spectroscopy. The content of hemoglobin-oxygen complexes increased significantly only in children with lasting disease (more than 1 year); oxygen-binding capacity of hemoglobin is significantly changed, while its capacity to release oxygen remained unchanged. These changes were paralleled by alteration of hemoglobin affinity for oxygen. The area and content of hemoglobin were studied by laser interference microscopy. Hemoglobin content increased significantly in erythrocytes of patients with a more than 1-year history of type 1 diabetes mellitus. In these children, a significant inverse correlation between oxyhemoglobin fraction, oxygen binding capacity, and cholesterol content was found, this clinical parameter positively correlated with affinity for oxygen measured by Raman spectroscopy.

Key Words: *erythrocyte; type 1 diabetes mellitus; hemoporphyrine; Raman spectrum; laser interference microscopy*

Type 1 diabetes mellitus (DM1), developing usually in children and adolescents is caused by insulin deficiency. Despite the progress in the treatment of DM1, delayed complications of the disease (angiopathies, neuropathies, atherosclerosis, *etc.*) remain very serious problems. The role of erythrocytes in DM1 patients has received sufficient attention. The life-span of erythrocytes in this cohort of patients is shorter [5], their shape [7] and lipid composition [2] are modified, and the membrane deformability is reduced [6]. Enzymes activities are also changed: activity of Na^+,K^+-ATPase is lower in patients with DM1 than in normal subjects, while activity of acetylcholinesterase is higher [8]. Obviously, blood content of glycosylated hemoglobin (HbAlc) is an important diagnostic indicator of diabetes: HbAlc affinity for oxygen is significantly higher than that of the native Hb [10]. All these changes, no doubt, modify erythrocyte capacity to carry blood gases.

Biological Faculty, M. V. Lomonosov Moscow State University, Russia. ***Address for correspondence:*** mityanina31@mail.ru. V. A. Mityanina

We studied oxygen-binding characteristics of erythrocyte hemoglobin in children with DM1 of different duration.

MATERIALS AND METHODS

The function of erythrocyte hemoglobin was studied in 3 groups of children aged 2-16 years. Group 1 (control) consisted of 11 children (9 boys and 2 girls) without DM1. Group 2 consisted of 11 children (7 boys and 4 girls) suffering from DM1 for less than 1 year. Group 3 consisted of 12 children (7 boys and 5 girls) suffering from DM1 for more than 1 year. Blood (2 ml) was collected from the ulnar vein into sterile tubes with EDTA under aseptic conditions after overnight fasting. Blood samples and the results of clinical analyses, carried out on automated hematological (Abacus Junior B, Diatron) and biochemical (Chem Well Plus, Awareness Technology Inc.) analyzers were granted by the Russian Pediatric Clinical Hospital.

Raman spectroscopy was used in the study. This method shows changes in hemoglobin hemoporphy-

0007-4888/12/1534-0508

rine conformation, which makes it possible to evaluate changes in the oxygen binding capacity and the oxyhemoglobin/total hemoglobin proportion directly in native erythrocytes [1].

In order to evaluate the peak intensities by Raman spectroscopy, a glass capillary filled with whole venous blood was placed into the well and an argon laser beam (λ=473 nm, P=18-20 mW) was focused on it. Three capillaries were taken for each sample; each was evaluated separately, and the mean value was then calculated. The Raman spectrum of venous blood measured under these conditions at 800-1800 cm^{-1} (Fig. 1) was presented by a set of bands corresponding to the spectrum of its prosthetic group hemoporphyrin. Hemoglobin Raman spectra depended on stimulation of the porphyrine ring fluctuations and ligand–iron bond fluctuations in the heme. These values characterized the prosthetic group status and hence, the changes in the hemoglobin oxygen-binding characteristics [3].

The erythrocyte status was studied by laser interference microscopy (LIM) [11] *in vitro*. The method for preparation of the samples was described previously [4]. At least 80 cells per sample were examined. The images were processed by the FIJI software. Hemoglobin content was evaluated by the formula:

$$m_{Hb} = \frac{\rho_{Hb}}{(n_{hem}-n_m)} F_{mean} S,$$

where S was the cell area, F_{mean} is the mean value of a measured parameter – optical path difference proportional to the erythrocyte thickness [11], ρ_{Hb} is specific density of hemoglobin equal to 1.65 g/cm^3, n_{hem} is hemoglobin refraction index equal to 1.615, and n_m is plasma refraction index equal to 1.35.

The results were statistically processed by Statistica 6.0 software. Selective verification of the distribution normality was tested by the Shapiro–Wilk test. As the parameters sometimes did not fit the normal distribution, the statistical significance of differences in the groups was tested by nonparametric Mann-Whitney and Wilcoxon tests. Changes were considered significant at $p<0.05$.

RESULTS

Blood levels of glucose, HbAlc, and cholesterol differed significantly in the groups (Table 1). The levels of triacylglycerols, hemoglobin, and erythrocyte counts virtually did not change; however, there was a trend to reduction of erythrocyte count and to an increase of the mean level of hemoglobin in an erythrocyte. The acute inflammation phase index (erythrocyte sedimentation rate) was similar in all groups and within the normal range of values (N=2-12 $mm \times h^{-1}$).

The quality of samples was evaluated by LIM before Raman spectroscopy. High aggregation of erythrocytes was found; however, this did not interfere measurements. A typical erythrocyte image is presented in Fig. 2. Cell levels of hemoglobin and cell area measured by LIM are presented (Table 2). Hemoglobin content was significantly higher in children with DM1 of more than 1 year duration. This was in line with the trend to an increase of hemoglobin levels in erythrocytes of similar patients, measured by the hematological analyzer (MCH value in Table 1).

Changes in the ratio of scatter intensities for respective erythrocyte hemoglobin hemoporphyrine spectrum bands were studied by Raman spectroscopy

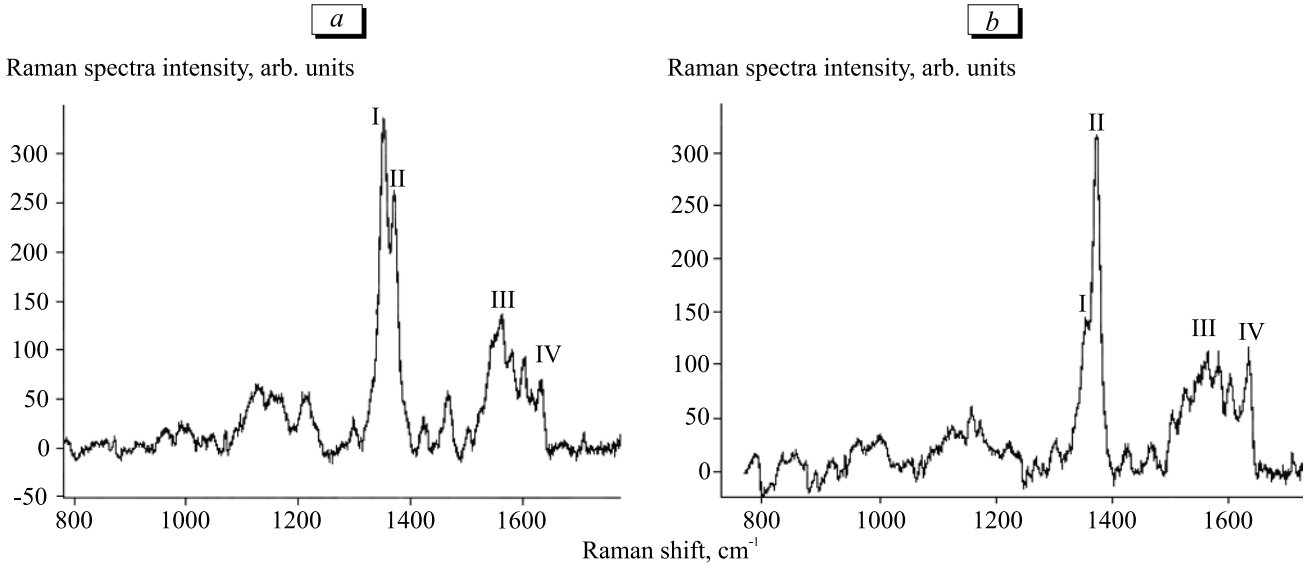

Fig. 1. Raman spectra of venous blood from a child without DM1 (*a*) and a child suffering from DM1 for more than 1 year (*b*). The maxima of the main peaks: 1355 cm^{-1} (I), 1375 cm^{-1} (II), 1550 cm^{-1} (III), and 1580 cm^{-1} (IV).

TABLE 1. Clinical Characteristics of Patients' Groups ($M\pm\sigma$)

Parameter	Group 1 (control; *N*=11)	Group 2 (DM1<1 year; *N*=11)	Group 3 (DM1>1 year; *N*=12)
Gender (m:f)	9:2	7:4	7:5
Age, years	7.3±3.2	8.1±4.5	11.0±3.7
Disease length, years	-	0.43±0.24*	3.6±2.8*
Glucose, mmol×liter^{-1}	4.7±0.6	10.2±3.4*	11.4±5.5*
HbA1c, %	5.9±0.3	8.1±2.4*	8.6±1.9*
Cholesterol, mmol×liter^{-1}	3.6±0.8	4.5±0.9*	4.8±0.8*
Triglycerides, mmol×liter^{-1}	1.1±0.3	1.0±0.8	0.9±0.6
ESR, mm×h^{-1}	4.8±1.9	5.7±2.0	5.3±2.5
Hemoglobin, g/liter	133.2±11.6	130.0±14.1	133.2±14.4
Erythrocytes, ×10^{12}	5.0±0.4	4.7±0.4	4.7±0.5
MCH, pg	26.9±1.9	27.7±1.6	28.8±0.9*
MCV, fl	80.1±4.5	81.4±4.0	84.4±2.8

Note. *p<0.05 in comparison with the control (Mann–Whitney's test). MCH: mean content of hemoglobin in an erythrocyte; MCV: mean erythrocyte volume; ESR: erythrocyte sedimentation rate.

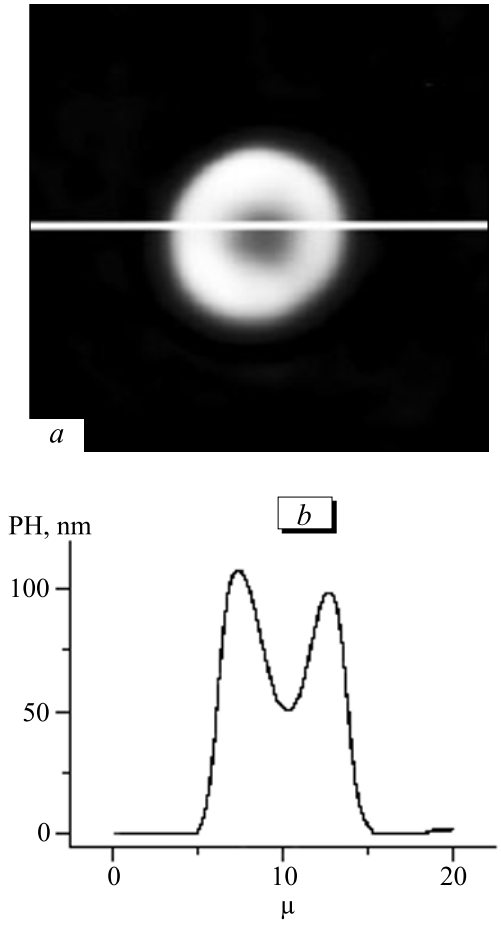

Fig. 2. Phase images (*a*) and profiles of changes in phase height (PH, *b*) in patients' erythrocytes.

in children with DM1 of different duration in comparison with controls. The intensities of spectrum bands 1355 and 1375 cm^{-1} were linked with symmetrical fluctuations of pyrrol rings in deoxyhemoglobin and ligand-bound hemoglobin molecules, respectively [9]. As the quantity of O_2 in the blood was by 3-4 orders of magnitude higher than the content of other ligands (for example, NO or CO), the intensity of band 1375 cm^{-1} was determined mainly by oxyhemoglobin content. Hence, the intensity proportion $I_{1375}/(I_{1355}+I_{1375})$ was proportional to oxyhemoglobin fraction in the blood. The intensities of bands 1550 cm^{-1} and 1580 cm^{-1} characterized the spin status of iron in the deoxy- and hydroxy forms, respectively, and hence, could be regarded as markers evaluating the structural characteristics of iron in the prosthetic group. Hence, using the I_{1355}/I_{1550} and I_{1375}/I_{1580} band proportions we can evaluate the capacities of erythrocyte hemoglobin molecule to bind and release oxygen molecules in accordance with the inner status of hemoglobin molecules. Dividing one proportion by the other $(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$, we derive a characteristic reflecting hemoglobin molecule affinity for oxygen in native erythrocytes.

Oxygen binding to hemoglobin and constant of erythrocyte hemoglobin affinity for oxygen decreased in children with DM1 for a long period in comparison with controls, while oxygen release by hemoglobin remains unchanged (Fig. 3). These shifts are paralleled by an increase of the content of hemoglobin-ligand complexes (mainly oxyhemoglobin) in the venous blood. Our study has shown an increase of oxyhemoglobin content only in children suffering from DM1 for a long period; this

TABLE 2. Characteristics of Patients' Groups Measured by LIM ($M \pm \sigma$)

Parameter	Group 1 (control; N=7)	Group 2 (DM1<1 year; N=5)	Group 3 (DM1>1 year; N=7)
Cell area, μ^2	51.5±5.6	50.9±1.4	54.8±3.3
Hemoglobin content, pg	26.0±4.4	26.8±0.6	30.2±2.4*

Note. *p<0.05 n comparison with the control (Mann–Whitney's test).

TABLE 3. Correlations of Raman Spectroscopy Values with Age and Biochemical Values

Parameter of Raman spectrum	Group 1 (control; N=7)		Group 2 (DM1<1 year; N=5)	Group 3 (DM1>1 year; N=7)
	area	hemoglobin (LIM)	Hematocrit	cholesterol
$I_{1375}/(I_{1355}+I_{1375})$	-	-	-	-0.85
I_{1355}/I_{1550}	-	-	-	-
I_{1375}/I_{1580}	-0.73	-0.76	-0.73	-0.75
$(I_{1355}/I_{1550})/(I_{1375}/I_{1580})$	-	-	-	0.93

Note. Significant coefficients of correlations (p<0.05) between peaks of Raman spectrum and other clinical parameters and values measured by LIM calculated using Statistics 6.0 software are presented.

was paralleled by a significant drop of hemoglobin capacity to bind oxygen. These changes were paralleled by a decrease of hemoglobin affinity for oxygen.

Hemoglobin affinity for ligands reduced in DM1. It seemed to be caused by a significant reduction of hemoporphyrine binding to ligands and a trend to greater release thereof. Type 1 DM is associated with metabolic acidosis of the blood, this also reducing hemoglobin affinity for ligands.

Correlations of Raman spectra parameters with age and biochemical values were evaluated. Strong and medium (surpassing 0.7 by modulus; Table 3) correlations were derived. Significant negative correlations between hemoglobin content (area) and capacity to release oxygen were found in the controls. A negative correlation between hematocrit value and cell hemoglobin release of oxygen was found in children suffering from DM1 for less than 1 year. There was no significant increase in the cell count between the groups, and hence, correlation between the increase of hematocrit value and increase of hemoglobin content in an erythrocyte could be traced. Hence, increase of hemoglobin level in an erythrocyte led to reduction of hemoglobin release of oxygen.

Children suffering from DM1 for more than 1 year developed a significant correlation between hematocrit value and hemoglobin affinity for oxygen. The increase of hemoglobin content led to increase of hemoglobin affinity for oxygen (similarly as in patients with DM1 of less than 1 year). In addition, children suffering from DM1 for more than 1 year developed a significant correlation between blood levels of triglycerides and cholesterol and Raman spectra parameters. Increase of blood cholesterol level in this group correlated with reduction of oxyhemoglobin fraction in

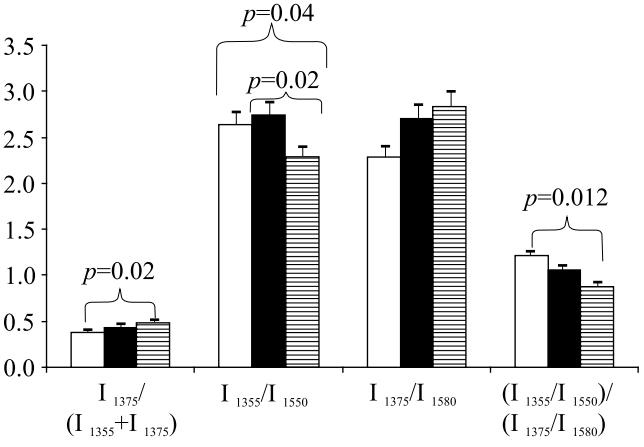

Fig. 3. Changes in the intensity proportions of characteristic bands of erythrocyte hemoglobin Raman spectra in children without DM1 (light bars) and children suffering from DM1 for less than 1 year (dark bars) and longer than 1 year (cross-hatched bars). The $I_{1375}/(I_{1355}+I_{1375})$ proportion reflects oxyhemoglobin content in the blood; I_{1355}/I_{1550} proportion reflects hemoglobin binding of ligands (including O_2); I_{1375}/I_{1580} proportion reflects hemoglobin release of ligands; and the $I_{1355}/I_{1550})/(I_{1375}/I_{1580})$ proportion shows hemoglobin affinity for ligands, primarily for O_2.

erythrocytes and a decrease of oxygen release and of hemoglobin affinity for oxygen, presumably because of increase of oxyhemoglobin fraction.

Hence, oxyhemoglobin content increased significantly only in children with DM1 lasting for more than 1 year; this was associated with a drop of oxygen binding by hemoglobin, its capacity to release oxygen remaining unchanged. These changes were paralleled by changes in the constant of hemoglobin affinity for oxygen and an increase of cholesterol level. This correlation indicated that changes in the microcirculation and in erythrocyte membrane structure played the key role in modification of the oxygen transporting activity of the blood in DM1.

REFERENCES

1. N. Yu. Bryzgalova, N. A. Brazhe, A. I. Yusipovich, *et al.*, *Biofiz. Kletki*, **54**, No. 3, 442-447 (2009).
2. D. A. Korzhenevskii, V. N. Kuptsov, V. A. Mityanina, *et al.*, *Mass-Spektrometriya*, **7**, No. 3, 188-192 (2010).
3. K. R. Solovyov, L. L. Gladkov, A. S. Starukhin, *et al.*, *Porphyrine Spectroscopy: Fluctuating States* [in Russian], Minsk (1985).
4. A. I. Yusipovich, N. Yu. Bryzgalova, E. Yu. Parshina, *et al.*, *Byull. Eksp. Biol. Med.*, **145**, No. 3, 357-360 (2008).
5. V. Chandramouli and J. Carter, *Diabetes*, **24**, No. 3, 257-262 (1975).
6. M. Garnier, J. R. Attali, P. Valensi, *et al.*, *Metabolism*, **39**, No. 8, 794-798 (1990).
7. H. Jin, X. Xing, H. Zhao, *et al.*, *Biochem. Biophys. Res. Commun.*, **391**, No. 4, 1698-1702 (2010).
8. L. Mazzanti, E. Faloia, A. Rabini, *et al.*, *Clin. Biochem.*, **25**, No. 1, 41-46 (1992).
9. O. V. Rodnenkov, O. G. Luneva, N. A. Ulyanova, *et al.*, *Pathophysiology*, **11**, No. 4, 209-213 (2005).
10. M. De Rosa, M. Sanna, I. Messana, *et al.*, *Biophys. Chem.*, **72**, No. 3, 323-325 (1998).
11. A. I. Yusipovich, E. Y. Parshina, N. Y. Bryzgalova, *et al.*, *J. Appl. Phys.*, **105**, No. 10, 102,037 (2009).