Journal of Fluorescence, Vol. 14, No. 1, January 2004 (©2004)

Use of the Fluorescent Probe DSM in Studies of the Structural and Functional Changes of the Erythrocyte Membrane

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Received June 30, 2003; revised August 21, 2003; accepted August 22, 2003

We have investigated the binding of *n*-toluenesulfonate-4-(*n*-dimethylaminostyrene)-1-methylpyridinium (DSM) to human and animal erythrocyte membrane. It was discovered the spectral characteristics and binding parameters of the probes differ in the norm and in various pathologies, at changing adrenoreactivity of the animal organism. It is concluded that the probe can be used to assess the changes in membrane properties and in the β-adrenoreceptive function of both the erythrocytes and the organism as a whole.

KEY WORDS: Fluorescent probe; erythrocytes; diagnostics.

INTRODUCTION

Analysis of the structural-functional organization of cell membranes, specifically, the studies of the functional state of β-adrenoreceptors is a topical problem in medicine and biology. Earlier works have shown the unidirectivity of the changes in the adrenoreceptive properties of various tissues and organisms, red blood cells, including during the pharmacological action on the andrenergic mechanisms of regulation in an organism [1,2]. Thus, red blood cells are a convenient test object which makes it possible to judge about the changes in the adrenoreactivity of an organism as a whole. Cell and membrane studies make extensive use of fluorescent probes [3,4]. The membrane probe–cation DSM (*n*-toluenesulfonate-4-(*n*-dimethylaminostyrene)-1-methylpyridinium) possesses such an advantageous combination of properties as low toxicity for cells, considerable increase of the fluorescence quantum yield during its binding to cell structures. Fluorescence of DSM is sensitive to the change in polarity, viscosity of the medium. There are also data in the literature on polychromaticity of the properties of DSM [4,5]. In this context, we used the probe DSM in studies of the structural-functional properties of human and animal erythrocyte membrane.

MATERIALS AND METHODS

Study Subjects

Six groups of humans were examined: Group 1 comprised of 10 healthy (at the age of 39 on the average, including 3 women); Group 2 comprised of 13 patients suffering cardiac infarction, during the first 3 days of their illness (at the age of 62 on the average, including 3 women); Group 3 comprised of 10 patients suffering unstable stenocardia (at the age of 59 on the average, including 4 women); Group 4 comprised of 13 patients suffering neurocirculatory dystonia of hypertensive type (at the age of 21 on the average, including 2 women); Group 5 comprised of 15 patients suffering chronic alcoholism of Stage 2 (at the age of 35 on the average, all men); and Group 6 comprised of 9 patients suffering chronic alcoholism of Stage 2 with alcohol withdrawal syndrome, all men).

Tests of the change in the adrenoreactivity were performed on guinea pigs.

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Changing the Adrenoreactivity of the Animal's Organism

Increased adrenoreactivity was due to intraperitoneal injection of guanethidine (30–50 mg/kg, 24 hr) [6], decreased adrenoreactivity was caused by intraperitoneal injection of propranolol (5 mg/kg, 20 min) [1]. The preparations were manufactured by the company VEB Arzneimittelwerk, Germany.

For the purpose of control, animals subjected to intraperitoneal injection of physiological salt solution were used. Blood was obtained by decapitation of animals.

Preparation of Erythrocyte Suspension

The erythrocytes were singled out from fresh blood by high-speed centrifugation at 1700 *g* centrifugal acceleration, followed by three-times washing of the precipitate with isotonic phosphate buffer [7]. Thereafter, 0.5 mL of the erythrocytes precipitate were suspended in 3.7 mL of the same buffer, thus obtaining a suspension featuring 10% hematocrit.

The Fluorescent Probe DSM

The fluorescent probe (*n*-doluensulfonate-4-(*n*-dimethylaminostyrene)-1-methylpiridinium (DSM) were synthesized at the Institute for Organic Chemistry, Latvian Academy of Sciences [4,5].

Sample Preparation and Fluorescence Measurements

The erythrocyte's suspension was incubated with DSM (concentration in sample 1,1-11.6 μkM at room temperature for 2 min. The time interval (1 hr) between cell isolation and the measurement of fluorescence was constant for all samples. Fluorescence parameters were registered on a Signe-4M (Latvia) spectrofluorimeter at excitation (λ_{ex}) wavelenght of 470 nm and emission (λ_{en}) wavelenght of 520–700 nm. Fluorescence intensity (F) was measured in arbitrary units (a.u.).

Determination of the Binding Parameters of the Fluorescent Probe DSM

To investigate the properties of cell membranes, we determined the following parameters of probe binding: concentration of bound probe R; binding constant K; number of binding sites N; intensity in the maximum of the shortwave band of the probe fluorescence in erythrocyte suspension; the ratio of intensities of the fluorescent probes in the maxima of shortwave and longwave bands (560–565 and 600–610 nm).

The amount of erythrocyte-band probe (expressed in μmol per L of eryhrocyte suspension) was determined as the difference between the total amount of the probe added to 1 mL of erythrocyte suspension and that of free probe. The content of free probe in the supernatant was calculated by the calibration curve of the dependence of fluorescence intensity on the concentration of the probe added to the supernatant.

Parameters K and N were determined by the graphic method of Klotz [8].

Statistical Analysis

Data are expressed as means $\pm$SD. Differences among groups were analyzed using the Mann–Whitney U test [9]. Regression analysis was performed to evaluate the independent reactions between parameters. A two-tailed value $p \leq 0.05$ was considered as statistically significant.

RESULTS

Spectral Characteristics of DSM

The maxima of the excitation spectrum for the probe in buffer (pH 7.0) was observed at 450 nm; at the emission of fluorescence, at 620 nm. In the spectrum of fluorescence of probe DSM in erythrocyte suspension we revealed two bands of luminescence of the erythrocyte-bound probe—with the maxima within 560–565 nm and 610–620 nm. Each of those bands had its excitation maximum (at 470 and 495 nm, respectively) (Fig. 1). Such shape of the spectrum is due to two different chromophores formed during the sorption of the probe on the outer surface of the erythrocyte membrane and in the more hydrophobic region of the lipid bilayer [4,5]. The fluorescence of the probe in the red region of the spectrum contributes to the high analytical sensitivity of the method.

Studies of the Properties of Cell Membranes Using Fluorescent Probe

To investigate the properties of cell membranes, we determined the binding parameters of the probes in the following cases.

1. At various diseases accompanied by disturbed states of cell membranes (cardial infarction, neurocirculatory dystonia by the hypertensive type, instable stenocardia, stage 2 chronic alcoholism,

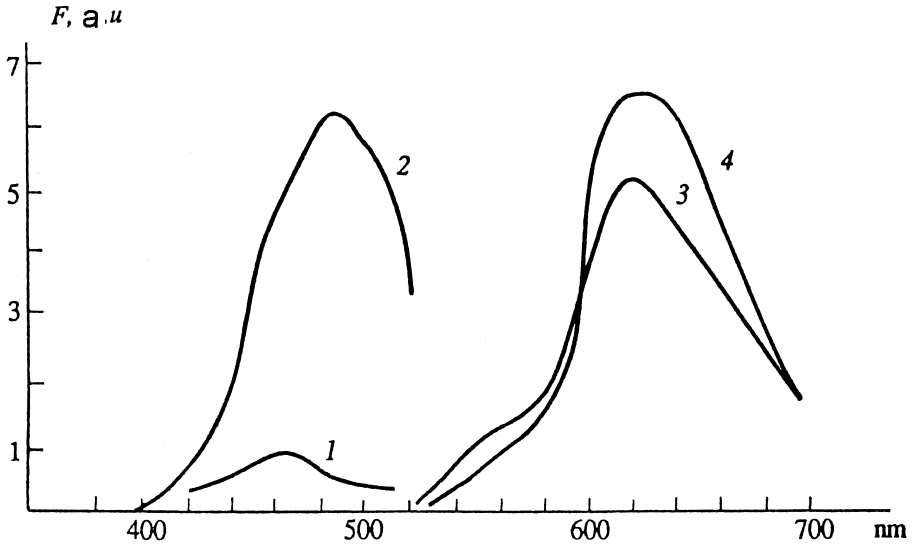

Fig. 1. Excitation (1, 2) and emission (3, 4) spectra of DSM fluorescence in erythrocyte suspension. The emission spectra were obtained at λ_{ex}470(3) and 495(4) nm; the excitation spectra were recorded at λ_{em}560(1) and 640(2) nm. Concentration of the probe, 50 μM, hematocrit 10%, monochromator slits, 2.86 nm. The fluorescence spectra was measured at room temperature.

stage 2 chronic alcoholism with alcohol withdrawal syndrome, etc.).

2. At changing adrenoreactivity of the animal organism (guinea pigs). We investigated the dependence of concentration of the probe bound to eryhrocytes (b) on concentration of the free probe in a supernatant (c) according to Klotz (the dependence of 1/b on 1/c; Fig. 2), which is widely used in investigations of ligand's interaction with matrix, allowing to judge on a nature of binding [9].

It was demonstrated that for all groups of the examined patients (except for one group comprised of the patients suffering with chronic alcoholism) concentration of the probe bound to erythrocytes is lower than for the healthy persons (Table I). Thus, the dependence of concentration of DSM bound to erythrocytes (b) on concentration of the free probe in a supernatant (c) for both the healthy persons and the examined groups of patients is linear in Klotz's coordinates (Fig. 2A), and, therefore, no judgement can be made on existence of one or several types of ligand's binding [8]. In order to elucidate this question, the curves of dependence of lg b/a and lg c/a on lg a were constructed, where a is concentration of the probe added to erythrocyte suspension (Fig. 3) [10].

Intersection of the curves 2 and 2′, 3 and 3′, 5 and 5′, as well as 6 and 6′ is indicative of the presence on erythrocyte membrane with patients suffering with ischemic cardiac disease, and also with those suffering with chronic alcoholism, sites of two types—featuring high constant of the probe binding ("specific") and low constant of such binding ("non-specific"). The curves, characterizing the patients suffering with neurocirculatory distonia, do not intersect, and this allows to conclude that in

Table I. Parameters of DSM Binding to Erythrocyte Membrane from Healthy Subjects and Patients (M±m)

No	Diseases	b, μM	K, μM^{-1}	N, μM	$K_{nsp} \cdot N_{nsp}$
1	Control (healthy subjects)	4.76 ± 0.12	2.00 ± 0.06	1.40 ± 0.05	0.32
2	Myocardial infarction	2.80 ± 0.22	0.25 ± 0.07	2.41 ± 0.06	0.62
3	Instable stenocardia	3.41 ± 0.27	0.75 ± 0.07	1.58 ± 0.07	0.66
4	Hypertensive type neurocirculatory distonia	3.12 ± 0.26	0.50 ± 0.07	1.71 ± 0.07	
5	Stage 2 chronic alcoholism	3.93 ± 0.16	1.12 ± 0.03	1.64 ± 0.03	0.89
6	Stage 2 chronic alcoholism with alcohol withdrawal syndrome	4.10 ± 0.42	1.46 ± 0.11	1.64 ± 0.06	0.93

Note. Concentration of the probe added, 8.05 μM. Parameters of particular groups were found to have significant differences ($p \leq 0.05$), with the exception of groups 1–6, 3–4, 3–6, 4–6, 5–6 (for b), 1–3, 3–4, 3–5, 3–6, 4–5, 4–6, 5–6 (for N) and 2–3, 5–6 (for $N_{nsp} \cdot K_{nsp}$).

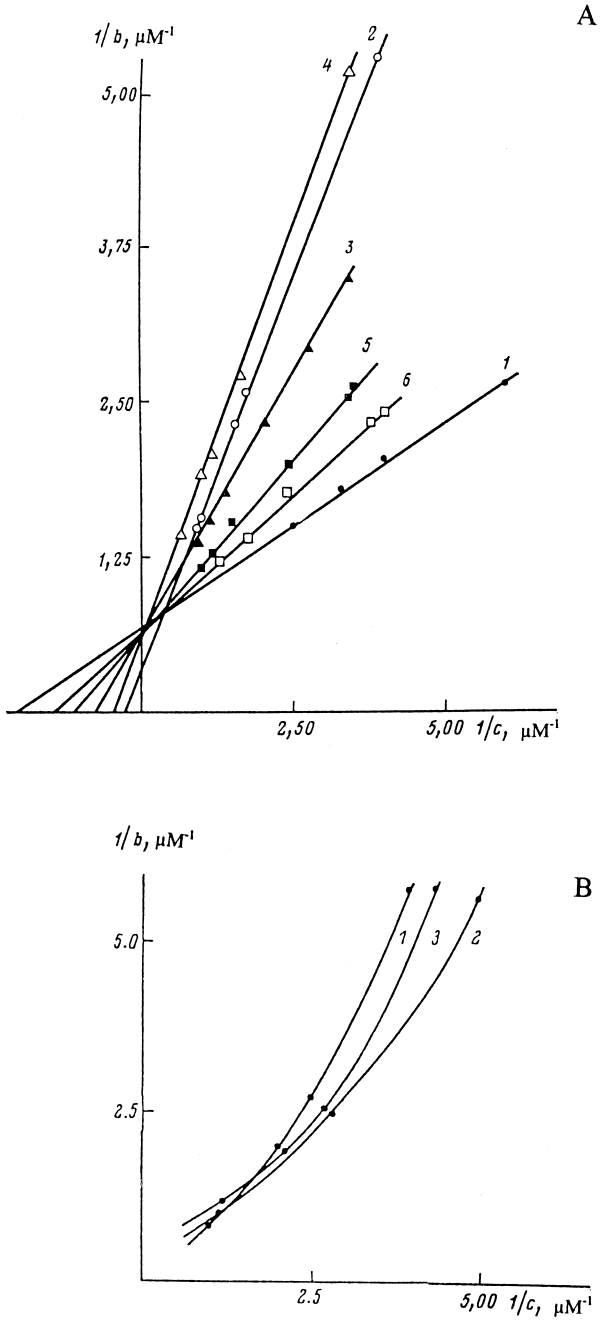

Fig. 2. Dependence of concentration of the erythrocyte-bound DSM (b) on concentration of the free probe in supernatant above erythrocytes in Klotz's coordinates. (A) numbers at curves indicate groups of patients (Table I). (B) 1) intact animals; 2) animals, subjected to intraperitoneal injection of guanethidine; 3) those subjected to intraperitoneal injection of propranolol.

such cases only one type of binding sites is present on the membrane.

Furthermore, by applying Klotz's graphical method, we determined the average values of binding constant (K)

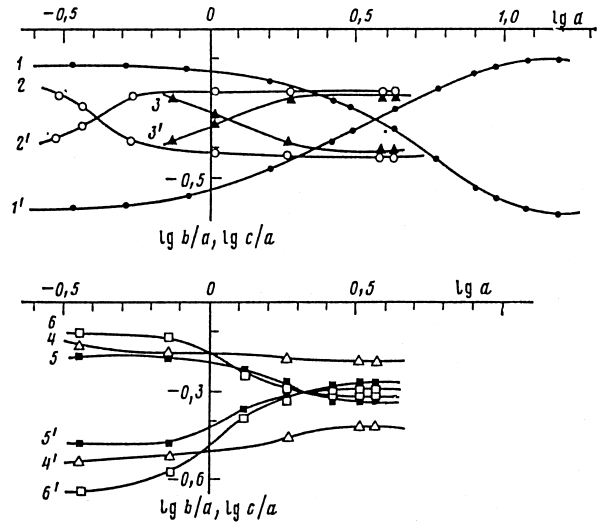

Fig. 3. Dependence lg b/a (1–6) and lg c/a (1'–6') on lg a, where a is concentration of the added probe, b is concentration of the bound probe, c is concentration of the free probe in supernatant. Numbers at curves indicate groups of patients (Table I).

and of count of DSM binding sites (N) (Table I). Analysis of the data shown allowed to establish that binding of DSM to erythrocytes of all examined patients is characterized by reduction of K and increase of N compared with the same parameters obtained for erythrocytes of healthy individuals. An exception is the data regarding concentration of binding sites for patients with instable stenocardia, which practically do not differ from the same indices showed by healthy individuals.

An attempt was made to determine parameters of DSM binding to areas of different types. With the help of a graph shown on Fig. 2, we discovered the value of the $K_{nsp} \cdot N_{nsp}$, which characterizes the probe binding to non-specific sites.

It was found that the index $K_{nsp} \cdot N_{nsp}$ increases with patients suffering with ischemic cardiac disease and chronic alcoholism, this being indicative of increase in concentration of the probe binding to non-specific sites with these patients. It turned to be impossible to determine parameters of the probe binding to specific sites for variants of the test due to nature of the curves lg b/a and lg c/a at small values of a [10].

In order to assess the β-adrenoreceptory function of erythrocyte membranes, we investigated the DSM probe binding under conditions of adrenoreactivity changed in vivo.

Changes in intensity (F) of the two bands of fluorescence of the probe bound to erythrocytes (λ_{max} = 560 and 615 nm) depending upon the level of adrenoreactivity of organism are shown in Table II. Depending on adrenoreactivity of an organism, the probe's fluorescence

Table II. Changes in Parameters of DSM Binding to Erythrocytes After Injection of Guanethidine and Propranolol to Guinea Pigs

Variant	K, μM^{-1}	N, μM	$K_{nsp} \cdot N_{nsp}$	N_{sp}, μM	K_{sp}, μM^{-1}	F(615 nm) a.u.	F(560 nm) a.u.
Control	0.072 ± 0.003	29.4 ± 1.5	0.76	1.99	0.47	6.0 ± 0.1	5.0 ± 0.1
Guanethidine	0.051 ± 0.003^a	50.0 ± 1.7^a	0.70	2.82^a	0.32^a	7.0 ± 0.1^a	4.7 ± 0.3
Propranolol	0.095 ± 0.005^a	21.8 ± 1.1^a	0.79	0.32^a	2.09^a	4.9 ± 0.1^a	4.9 ± 0.3

Note. Increased adrenoreactivity was due to intraperitoneal injection of guanethidine 930–50 mg/kg, 24 hr); decreased reactivity, by intraperitoneal injection of propranolol (5 mg/kg, 20 min).

[a]The values reliably differing from those obtained in investigation of erythrocytes of intact animals ($p \leq 0.05$).

in 615 nm band changed, this being indicative of increase in concentration of the bound probe. No significant difference of the probe binding indices was found, judging by its fluorescence in 560 nm band, compared with control results.

We investigated the dependence of concentration of the probe bound to erythrocytes (b) on concentration of the free probe in a supernatant above erytrocytes (c) according to Klotz. In Klotz's coordinates (Fig. 2B) these data are expressed by concave curves. Interaction of the DSM probe with erythrocyte membranes, judging by the data obtained, is characterized irrespective of the changes in adrenoreactivity of organism with positive cooperativity and heterogeneity of the binding sites.

By applying Klotz's graphical method, we determined the average values of binding constant (K) and of count of binding sites (N). It should be noted that the values of parameters K and N under increased and decreased adrenoreactivity of animals differ from the respective parameters of control group (Table II).

The existence of two types of binding, namely, specific and non-specific, is confirmed also by the appearance of curves in Fig. 4.

With the help of this graph, we determined the values of concentration of binding sites (N) and binding constant (K) in order to find the value of the $K_{sp} \cdot N_{sp}$ for the specific type of the probe binding, and that of the $K_{nsp} \cdot N_{nsp}$ for the non-specific type. The respective data are shown in Table II. It was discovered that at heightened adrenoreactivity of organism the concentration of specific binding sites N_{sp} increases, and at lowered adrenoreactivity of the same the concentration of specific binding sites N_{sp} decreases, while constant of specific binding K_{sp} increases. Thus, it was demonstrated that concentration of the bound DSM, and parameters of the probe binding to erythrocyte membranes change depending on adrenoreactivity of the organism.

DISCUSSION

It is widely known that a complicate complex of biochemical changes, which predetermine development of various pathologies (cardio-vascular disorders, including also alcoholism), is first of all characterized by disturbances in lipid metabolism [11–13]. In various pathologies and extreme effect on the organism the system of the antioxidant protection of the lipids is enhanced, the share of cholesterol is increased, and the lipid set in the erythrocyte membrane is changed [11,12].

Increase of molar relation cholesterol/phospholipids in erythrocyte membrane with patients suffering with hypertonic and ischemic cardiac diseases leads to changes in physico-chemical properties of this membrane, in particular, to increase of micro-viscosity of lipid bi-layer, increase

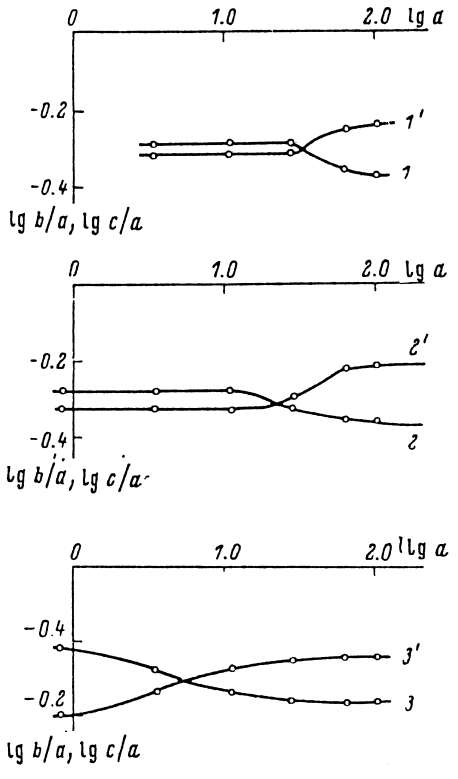

Fig. 4. Graphical determination of parameters of binding of DSM fluorescent probe to erythrocytes in guinea pigs. The designations are the same as on Fig. 3. (1) intact animals; (2) animals subjected to intraperitoneal injection of guanethidine; (3) those subjected to intraperitoneal injection of propranolol.

of its hardness and reduction of erythrocytes deformability [14–16]. It is known also that with patients suffering various forms of alcoholism structural-and-functional changes in erythrocyte and other cell membranes can be observed [13]. It is demonstrated that even at low concentrations of alcohol a disarrangement of hydrophobic part of membranes occurs, it is known also that at chronic use of alcohol the changes in lipid metabolism and their composition within membranes occur [17].

It should be noted that in most cases investigated, the change of parameter b correlates with the dynamics of changes in parameters N and F (560). These correlations appear to mean that the changes in the concentration of the erythrocyte-bound probe and parameters of binding are determined basically by the physico-chemical properties of the membrane lipids.

The changes in parameters of DSM binding to blood erythrocytes with patients, compared with erythrocytes of healthy individuals, are indicative of sensitivity of the probe to structural changes in erythrocyte membrane with the investigated diseases. The different degree of changes in parameters of the probe binding is testifying to different pronouncement of structural changes in erythrocyte membranes in the investigated pathologies (the largest differences among healthy individuals were detected with patients suffering ischemic cardiac disease and neurocirculatory distonia). Reduction of the values of b and K with patients suffering cardiac infarction, compared with those suffering instable stenocardia, confirms the data found in the literature, stating that disorders in structural organization of membranes directly depend on the gravity of pronouncement of ischemic cardiac disease.

Judging by the detected changes in DSM binding parameters depending on adrenoreactivity of animals, we can assume probability of binding of a part of the probe to β-adrenoreceptors. Such assumption can be confirmed also by the data obtained in experiments performed earlier on rats. Administration of guanethidine (50 mg/kg) decreased the noradrenaline content in adrenergic neurons, which led to an augmentation of the effector organ's adrenoreactivity in rats. The affinity of the erythrocytes' β-adrenoreceptors to propranolol was enhanced, and parameters of their binding the fluorescent probe were changed [18]. The data suggest the possibility of using erythrocytes for in vivo estimation of functional state of adrenoactive system in animals and humans.

A decrease in the affinity of the probe-cation to the membrane can also be indicative of decay in the efficient negative charge of the membrane. According to the literature, this is due to decrease of such indices of cell metabolism as activity of ATPase and other membrane-bound enzymes, a decrease in the consumption of the ATP energy pool [19].

It should be noted that the shifts of binding parameters of the probe in the pathologies studied are qualitatively similar with those observed in animals during experimentally enhanced adrenoreactivity of the organism.

The similarity in the character of membrane response to the change in the adernoreactivity of the organism in diseases of cardiovascular system suggests that along with structural rearrangement of the erythrocyte membrane the structural and functional properties of the adrenoreceptors also change.

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