EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Fluorescent Probing of Lymphocyte and Erythrocyte Plasma Membranes in Persistence of Tick-Borne Encephalitis Virus: Monitoring of Structural Changes

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The structure of lymphocyte and erythrocyte plasma membrane in patients with long persistence of tick-borne encephalitis virus was studied using fluorescent probes pyrene and 1-anilinonaphthalene-8-sulfonate. The authors analyze the clear-cut disorders in the membranes of both lymphocytes and erythrocytes in viral tick-borne-encephalitis from the viewpoint of generalized involvement of cell membranes in infectious process.

Key Words: *viral tick-borne encephalitis; lymphocyte; erythrocyte; plasma membrane; fluorescent probe*

Disease development is always associated with functional imbalance of this or that cell system. Dysfunction of one group of cells can be the main cause of the disease, while the function of other cells can be impaired as a result of progress of the underlying disease [7].

Pathological changes in cell homeostasis can be the cause or result of structural disorganization of its plasma membrane. Since cell membrane mediates active ion transport, cell-cell communication, interactions between the cell and environment, and other vital functions, molecular disorders of the plasma membrane in disease lead to loss of the functional competence, changes of cell viability, and even cell death [4].

The interest to membrane mechanisms of cell disorganization extended our notions on compensatory adaptive and dystrophic changes accompanying pathological processes of different origin. Many diseases are now considered as states associated with cell membrane disturbances [9,11]. On the other hand, available data on the properties of cell membrane compartment in infections, in particular in persistent viral infections characterized by long-term presence of the infectious agent in the body, are insufficient for evaluating the type and degree of involvement into the pathological process of cell systems to which the virus is tropic and cells which do not serve as targets for the virus [1, 2,10]. The aim of this study was to evaluate the structure of plasma membranes in cell systems (lymphocytes, erythrocytes) playing different roles in the realization of infectious process in carriers of tick-borne encephalitis (TBE) virus.

MATERIALS AND METHODS

A total of 33 patients with viral TBE (14 men, 19 women aged 23-50 years) were examined, 21 of these was asymptomatic TBE virus carriers and 12 patients

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had chronic manifest TBE. The disease was diagnosed in each case from the fact of the tick sucking, serological (indirect hemagglutination test and enzyme immunoassay) and molecular genetic (PCR) findings, and results of instrumental methods of examination. Patients with somatic diseases and other infections were not included in the study group. Control group consisted of 17 healthy donors of the same sex and age. Venous blood was stabilized with 25 U/ml heparin.

Erythrocyte membranes were isolated by the hypoosmotic method [14] and examined using fluorescent probes pyrene and 1-anilinonaphthalene-8-sulfonate (ANS; Sigma). Interactions of membranes with fluorescent probes were recorded on an MPF-4 spectrofluorimeter (Hitachi).

Microviscosity of the lipid phase of erythrocyte membrane was evaluated by eximerization of pyrene migrating into the hydrophobic compartment of the membrane in a medium containing (in mM): 145 NaCl, 10 Tris-HCl (pH 7.4) at excitation wavelengths (λ_E) 285 and 340 nm. Pyrene solution in ethanol was added to the cuvette with erythrocyte ghosts (protein content 0.3 mg/ml) to a final concentration of 10 μM and incubated for 10 min at constant stirring. Pyrene eximerization coefficient was estimated as the ratio of the maximum fluorescent intensities of eximer (470 nm; I_{470}) to monomer form (390 nm; I_{390}) [3,6].

In order to determine the parameters of ANS binding to the erythrocyte membrane 2 titrations were carried out. The first titration was carried out with different protein concentrations (from 0.3 to 0.6 mg/ml) at constant concentration of the probe (5 μM; bidistilled water served as the solvent); the second titration was carried out with different concentrations of the fluorophore (from 5 to 20 μM) at a constant concentration of protein (0.3 mg/ml). ANS fluorescence was excited at λ_E=360 nm, fluorescence intensity was measured at λ=490-510 nm. Binding constant and the number of ANS binding sites on the membrane were determined by the graphic method using inverse coordinates [3].

Lymphocyte suspension was isolated in a Ficoll-verografin density gradient (1.077 g/cm^3) [8]. The lymphocyte suspension was diluted to the concentration of 2×10^5 cells/ml with Hanks solution. The structure of lymphocyte plasma membrane was studied using pyrene probe. The probe in a concentration of 10 mM was added to the lymphocyte suspension and incubated for 20 min. The ratio of eximer to monomer fluorescence intensities (470 and 390 nm, respectively) was determined at λ_E=285 nm [6].

The significance of differences between the groups was evaluated using Student's *t* test and nonparametrical Mann-Whitney's test.

RESULTS

Viruses can destabilize cell membranes, which is the key stage in the process of membrane fusion with the viral particle [15]. The cytotoxic mechanism of the effects of some viruses on lymphocytes consists in impairment of membrane permeability [13]. On the other hand, virus reproduction in immunocompetent cells leads to functional and structural changes in lymphocytes [1]. Structural modification of lymphocyte plasma membrane occurs under conditions of viral damage of lymphocytes; it disturbs binding of second messengers and ligands to receptors, reduces activity of many membrane-associated enzymes, modulation of the lymphocyte receptor system, and eventuates in deficiency of lymphocyte immune functions [1, 2,12].

Study of the membrane structure in lymphocyte of patients with long-term persistence of TBE virus tropic to immunocompetent cells showed clear-cut modification of the lymphocyte membrane. Fluorescent probing of the lymphocyte plasma membrane with a nonpolar probe pyrene diffusing in the hydrophobic compartment of the membrane showed a significant decrease in the mean pyrene eximerization coefficient in patients with chronic TBE and asymptomatic carriers of TBE virus (1.38±0.08 and 1.40±0.09 arb. units, respectively, *vs.* 1.58±0.05 arb. units in

TABLE 1. Results of Studies of Erythrocyte Membranes by Pyrene and ANS Fluorescent Probes in Patients with Persistent Viral TBE (*X*±*m*)

Group	Pyrene fluorescence parameters		ANS fluorescence parameters	
	$\lambda_{470}/\lambda_{390}$, λ_E=285 nm, arb. units	$\lambda_{470}/\lambda_{390}$, λ_E=340 nm, arb. units	ANS binding constant, μM^{-1}	number of ANS binding sites, μmol×mg/ml
Control	0.310±0.012	0.360±0.015	0.126±0.014	0.653±0.078
Chronic viral TBE	0.233±0.012*	0.256±0.014*	0.231±0.031*	0.915±0.130**
Asymptomatic TBE virus carriership	0.266±0.022**	0.363±0.028+	0.191±0.023*	0.888±0.045**

Note. *p<0.001, **p<0.05 compared to the control; +p<0.05 compared to chronic TBE.

healthy subjects, $p<0.05$). Since the degree of pyrene eximerization is inversely proportional to membrane lipid phase viscosity, the observed decrease in this parameter in patients with persistent viral TBE attests to better ordering of lipid molecules in lymphocyte membrane [3]. Lymphocytes with increased microviscosity of the plasma membrane are characterized by low immunoreactivity [5].

However, the detected structural modification of the lymphocyte plasma membrane in chronic carriers of TBE virus was not specific for cells serving as targets for the virus. Similar changes in membrane structure were detected in fluorescent probing of the erythrocyte membrane. Erythrocyte membrane was selected as the object of investigation, because it is characterized by common principles of molecular organization of plasma membranes [4]. That is why the detected regularities in the structural and functional disorders of erythrocyte membrane can be extrapolated to other membrane systems with amendments for the species specificity of the cells.

Analysis of fluorescence spectra of deep lipotropic probe pyrene in erythrocyte membranes of patients with chronic viral TBE showed a clear-cut decrease of the mean eximerization of the fluorophore at λ_E=285 and λ_E=340 nm in comparison with healthy donors, which indicated increased microviscosity of the membrane lipid bilayer. Evaluation of pyrene eximerization coefficient in asymptomatic carriers of TBE virus showed an increase of the molecule orderliness in the annular lipid fraction (at λ_E=285 nm; Table 1). Study of the structure of surface layers of erythrocyte membrane using a negatively charged ANS probe showed that the erythrocyte membrane in patients with long-term persistence of TBE virus had several-fold more ANS binding centers than the red cell membrane in donors. This indirectly attested to changes un the charge of the phospholipid polar heads. A statistically significant increase in the mean constant of ANS binding to erythrocyte membrane in viral infection indicating higher affinity of the membrane for the probe was also worthy of note (Table 1).

These findings indicate pronounced restructuring of the lymphocyte and erythrocyte plasma membranes during long-term persistence of TBE virus, which suggests generalized damage to the plasma membranes during the development of infectious process. Presumably, the regularities of cell reaction to pathogen are common, irrespective of the cell type: any cell system functioning in accordance with its own laws develops similar structural and functional disorders, *e.g.* at the level of plasma membrane, under conditions of disease. We believe that these stereotypical changes in cell membrane structure are due to the fact that molecular mechanisms of membrane modification are largely universal and typical of different cell systems. Among these mechanisms are intensification of free-radical oxidation, activation of phospholipases and proteases, and imbalance of intracellular calcium homeostasis [7].

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