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ORIGINAL PAPER

Structural Changes in Lymphocytes Membrane of Chernobyl Clean-up Workers from Latvia

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Abstract ABM (3-aminobenzanthrrone derivative) developed at the Riga Technical University, Riga, Latvia) has been previously shown as a potential probe for determination of the immune state of patients with different pathologies .The fist study (using probe ABM) of peripheral blood mononuclear cells (PBMC) membranes of 97 Chernobyl clean-up workers from Latvia was conducted in 1997. Now we repeatedly examine the same (n=54) individuals in dynamics. ABM spectral parameters in PBMC suspension, fluorescence anisotropy and blood plasma albumin characteristics were recorded. In 1997 screening showed 5 different patterns of fluorescence spectra, from which in 2007 we obtained only two. These patterns of spectra had never been previously seen in healthy individuals or patients with tuberculosis, multiple sclerosis, rheumatoid arthritis, etc., examined by us. Patterns of ABM fluorescence spectra are associated with membrane anisotropy and conformational changes of blood plasma albumin. We observed that in dynamics 1997–2007 the lipid compartment of the membrane became more fluid while the lipid-protein interface became more rigid. The use of probe ANS and albumin auto-fluorescence allowed show conformational alterations in Chernobyl clean-up workers blood plasma. It is necessary to note that all investigated parameters significantly differ in observed groups of patients. These findings reinforce our understanding that that the cell membrane is a significant biological target of radiation. The role of the membrane in the expression and course of cell damage after radiation exposure must be considered. So ten years dynamic of PBMC membrane characteristics by ABM (spectral shift and anisotropy indexes) in Chernobyl clean-up workers reveal progressive trend toward certain resemblance with those of chronic B-cell lymphoid leukemia.

Keywords Fluorescent probe · Peripheral blood mononuclear cells · Membrane anisotropy · Proteins

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Introduction

Membrane damage is considered to play a key role in the killing of cells and in the loss of many different membrane functional properties induced by ionizing radiation. [1–3]. Cells may be damaged, rather than killed, at low dose ranges, but membrane damage may be generally detrimental to the life of the cell. Studies of the structural and functional relationships in biological membranes have shown that many immunological functions may be heavily dependent on the cell structure [4, 5].

Particular, maintenance of the structure and function of various cellular contacts provides homeostatic regulation of cell proliferation and differentiation and of the adaptive responses of individual cells.

Capacity of the biological membrane to control the cell interface, the transmission of intercellular signals, and subsequent metabolic pathways depends on its structural integrity in the maintenance of its biophysical properties. This

capacity underlies the pivotal role of the membranes in the expression and course of cell damage after radiation exposure.

The aim of this work was to determine the alteration of membrane dynamic parameters of peripheral blood mononuclear cells (PBMC) of Chernobyl clean-up workers by using the fluorescent probe ABM.

Materials and methods

Study subjects

The study subjects were randomly selected from Latvia's residents (male, n=97) who had participated in the accident cleaning-up works in Chernobyl. For the first time their PBMC were examined by fluorescent probe ABS in May–June 1997, and repeatedly the same individuals (n=54) were tested during April 2006–June 2007.

Individuals who had been under treatment for acute infections, active autoimmune diseases malignancy were excluded.

Control group (n=17, male) consists from the healthy individuals of corresponding age, who never had professional contact with radioactivity.

Blood collection

The Ethics Committee of the Medical Academy of Latvia approved the protocol of the present study design.

Peripheral blood samples for PBMC studies were collected from vein into disposable vacuum tubes containing preservative-free heparin 30 IU ml^{-1}.

The fluorescent probe ABM

ABM (conditional name) was synthesized at the Riga Technical University Department of Organic Chemistry [6].

Synthesis, properties and research results to determine the immune state of patients, at different pathologies have been summarized in [7]. It was established that ABM spectral parameters reflect a wide range of interrelated (interdependent) characteristics of PBMC: physicochemical state and micro-viscosity of membrane, proliferate and lipid metabolic activity of cells, distribution of lymphocyte subsets [7]. The observed changes of the studied ABM spectral parameters reflect alterations of the cellular mechanisms of immunity which is a main focus for its applications as preliminary screening test in immune diagnostics [7].

PBMC preparation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh withdrawn heparinised venous blood by standard verographine method. Three ml of blood were layered on 3 ml of verographine and centrifuged at 2500 rpm for 30 min (60% verographine Spofa:aqua pro inj., 2:5). The ring PBMC formed on the density interface was aspirated and washed 3 times with potassium buffer solution (pH 7.3) by centrifugation at 1500 rpm for 10 min., then re-suspended in RPMI 1640. The resulting PBMC suspension containing $.5\times10^6$ cells per ml was applied for the fluorescence measurements.

Sample preparation and fluorescence measurements

Investigations were performed by means of the newly synthesized fluorescent probe ABM. The fluorescent dye ABM was dissolved in ethanol, with a resulting 0.1 % concentration of ethanol in probe. The "blank" sample for each experiment was prepared by ABM titration without cells. The PBMC suspension was incubated with 19.6 $nmol/1^{-1}$ ABM (resulting concentration) at room temperature for 5 min. The interval (3 hours) between cell isolation and the fluorescence measurement was constant for all samples. Fluorescence parameters were registered on spectrofuorimeter Spectrofluo JY3 (ISA Jobin Yvon Instruments S.A., France) at excitation wavelength 470 nm and emission wavelength of 520−700 nm. Fluorescence intensity (F) was measured in arbitrary units (F, a.u.).

Measurements of fluorescence polarization

The fluorescence anisotropy was detected using two probes—ABM and ANS (8- aniline-1-naphtalene sulfuric acid). ABM and ANS solutions were each added to cell suspensions to final concentrations of 19.6 nmol/ml and 10 nmol/ml, respectively, with cell labeling achieved after incubation for 5 and 20 min at 18–20 C^o, respectively.

ABM fluorescence parameters were registered at excitation wavelength 470 nm and emission wavelengths 610 nm (Group 1) and 635 nm (Group 2). For ANS excitation wavelength was 393 nm, emission wavelength was 470 nm [8].

In each case, non-probe-labeled cell suspension (blanks) was measured before each series of experiments.

Measurements of fluorescence anisotropy

The anisotropy index "A" was calculated according to [9].

Statistical analysis

Statistical differences among groups having different spectral characteristics were calculated using the Student's *t*-test and Whitney–Mann's U test [10].

Results and dicussion

ABM spectral characteristics in PBMC suspension

The first examination of these patients was performed in 1997 (11 years after they participated in the cleaning-up of the Chernobyl accident aftereffects). Screening of individuals showed 5 patterns of ABM fluorescence spectra [7, 11]. The emission maximum of ABM in PBMC suspension for healthy donors is 630 nm.

Screening of the individuals in April 2006–June 2007 (20 years after the work in Chernobyl) showed the following two patterns of fluorescence spectra (Table 1).

1. Fluorescence zone is shifted to the short-wave region of spectrum by 10–50 nm (max 580–640 nm). The obtained shift is more remarkable than in 1997 (3–13 nm) and characterize 83% of observed patients (n=45; in 1997 n=12, 12%). Max shift on passing from 620 nm to 580 nm is accompanied by increasing of ABM fluorescence intensity: 1,3–4,0 times higher than that observed in healthy donors (see Table 1); in 1997 it was 2.1 times higher.
2. A wide fluorescence maximum (plateau) in the region of 625–650 nm was obtained only for 8 (15%) patients (in 1997 n=54, 56%). Fluorescence intensity is 2.3 times higher than that in healthy donors group (Table 1).
3. The fluorescence zone is shifted to the long-wave region of the spectrum by 25 nm (n=1) (in 1997 n=14, 14.4%).
4. There are no patients with ABM fluorescence maximum at 630 nm corresponding to the fluorescence observed in healthy persons (in 1997 n=17, 18%).

Table 1 Spectral characteristics of probe ABM in Chernobyl clean-up workers PBMC suspension anisotropy index, detected using probes ABM and ANS

Groups of patients	Maxima of emission, nm	F, a.u.	Anisotropy index	
			ABM	ANS
1A	580–589 nm (n=10)	3,17±0.18	–	–
1B	590–599 nm (n=9)	1.97±0.11	–	–
1C	600–620 nm (n=24)	1.28±0.07	0.233±0.05	0.220±0.06
2	625–650 nm (n=8)	1.74±0.11	0.219±0.05	0.196±0.04
3 Control	630 nm (n=17)	0.71±0.04	0.254±0.04	0.177v0.05
		1A–1B	1C-2;1C-3	1C-2; 1C-3
		1A–1C	2–3	2–3
		1B–1C		
		1A-2; 1C-2		
		1A-3; 1B-3		
		1C-3; 2-3		

ABM concentration in cell suspension 19.6 nm/ml
ANS concentration in cell suspension 10.0 nm/ml
p-differences between groups are significant (p>0.05)

Such spectral characteristics of PBMC of Chernobyl clean-up workers (the 1st and the 2nd pattern of spectra) obtained 20 years after the work in Chernobyl are due to ABM fluorescence originating from lipid-bound ABM and from protein-bound ABM.

Yonei and Kato [12] showed that changes in the fluidity of irradiated membranes were primarily due to conformational and biochemical changes of membrane proteins and/or lipids. The two obtained fluorescence patterns (Groups 1, 2) have never been seen in healthy individuals or in patients with lung diseases, multiple sclerosis, rheumatoid arthritis etc., which we tested previously using ABM [7, 13]. The emission spectra of PBMC-ABM of the patients represented zone of fluorescence without sign of fluctuating structure. The emission spectra and their wavelength maxima of these patients did not differ from those of healthy donors [7, 13]. The obtained two patterns of Chernobyl clean-up workers spectra suggest that various qualitative changes of membrane properties are evident in their PBMC as compared with previously examined healthy donors or patients having no professional contact with radioactivity [7, 13].

Fluorescence anisotropy and membrane fluidity

We looked at whether the modification of the oxidative status and the resulting structural change after radiation exposure might have affected the overall biophysical properties of the membrane, most specifically, its fluidity.

Fluorescence anisotropy data for cells are shown in Table 1.

Using two fluorescent probes ABM and ANS, the lipophilic phase of the membrane appeared more fluid whereas the lipid-protein interface of the membrane was shown be more rigid in observed patients as compared with those in healthy donors. These data suggest that the anisotropy variations are representation of the local properties of the fluorescent probes microenvironment [1, 2]. According to [8], ANS fluorescence emission results from the contributions of both proteins and lipids. These contributions depend on the ANS concentration. In our experimental conditions, i.e. low concentration of ANS, the fluorescent probe would bind preferentially to membrane protein [8].

It is interesting to note that fluorescence anisotropy value obtained significant differences in observed patients groups (see Table 1).

Several authors point out that radiation exposure leads to reorganization of the fatty acid pattern. The phospatidylethanolamine fatty acids (n=3) and (n-6) series decreased, while the phosphatidylethanolamine saturated fatty acid content increased [1–3].

Fluorescent probes could reveal all these modifications may be involved in the variation of the biophysical properties of the membrane in Chernobyl clean-up workers PBMC.

As mentioned above, the authors have previously shown that these effects are related to radiation induced lipid oxidative process [1].

It has been also established that fluorescent probe ABM binds to proteins: the observed changes in fluorescence characteristics corresponded to the known conformational transitions of albumin [14]. It is necessary to note, that albumin is a single source of ABM fluorescence in human blood plasma.

The significant ANS florescence anisotropy increase indicates membrane rigidification of the lipid–protein interface. This phenomenon could be related to lipid–protein binding or a protein conformational change. One hypothesis favours the formation of Shiff bases with primary amine functions carried by proteins or phospholipids, leading to lipid–protein or protein–protein cross-linking. Conformational changes of membrane proteins induced by ionizing radiation are described by Verma and Sonwalkar [15] using Raman spectroscopy. Their data demonstrate that the state of lipid–protein phase, the secondary structure of proteins and the environment of aromatic amino acids were altered in the plasma membrane.

Characterization of albumin in patients blood plasma

ABM binding data with blood plasma albumin and plasma albumin self-fluorescence for observed patients are shown in Table 2.

It has been established that fluorescent probe ABM binds to proteins: the observed changes in fluorescence characteristics correspond to the known conformational transitions of albumin [14].

Albumin is a single source of ABM fluorescence in human blood plasma (max 650 nm).

In the Group 1 the fluorescence zone is shifted to the short-wave region of spectrum by 20–50 nm.

The 2 Group had a fluorescence maximum at 650 nm corresponding to the fluorescence observed in healthy donors (Table 2). Fluorescence intensity in observed clean-up workers decreases (in Group 1 more remarkable as compared by healthy donors and Group 2).

These observations may be consistent with the ABM decreased binding and/or conformational changes of albumin molecule [16] significantly differing in patients groups. In previous experiments with human serum albumin are obtained that changes of pH in the range from 3to 12 strongly affect the fluorescence intensity and spectrum of albumin-bound ABM [14].

The most prominent changes in spectral characteristics occurred at pH values known to cause conformational transitions of proteins [8]. Data in Group 1 was similar to that obtained at pH 1–2: the fluorescence zone is shifted to the short wavelength region as compared to the spectrum at pH 7.4; fluorescence intensity decreases. According to the literature, the acidic expansion of albumin globule takes place [15, 16]. No so significant alterations in albumin structure in Group 2 was obtained as compared with Group 1 (Tables 1 and 2).

On excitation the human blood plasma with 286 nm, practically single source of fluorescence are tryptophanyl of albumin molecule. Obtained results may be explained by conformational changes of proteins. The shift of fluorescence zone to short-wave region (Group 1) is evidence of hydration of tryptophanyl region of albumin molecule [16]. Results is in agreement to ANS lower degree of polarization Group 1 patients (i.e. higher mobility of bound ANS, higher accessibility of water to these binding sites as compared with Group 2 (Table 1) [8].

Different patterns of spectra suggest that various qualitative changes of membrane properties are evident in PBMC of Chernobyl clean-up workers, in comparison with previously examined healthy donors or patients having no professional contact with radioactivity [6, 7, 13].

Obtained modifications may be involved in the variation of the biophysical properties of the membranes that we noted after radiation exposure. Specifically, we observed

Table 2 Spectral characterization of albumin in Chernobyl clean-up workers blood plasma

Groups of patients	Probe ABM		Albumin Auto Fluorescence	
	Fluorescence emission max, nm	F, a.u.	Fluorescence emission max., nm	F, a.u.
1C	600–630 nm	1.24±0.07	311–318 nm	2.15±0.06
2	650 nm	1.74±0.06	330 nm	2.48±0.04
3 Control	650 nm	2.11±0.06	330 nm	2.96±0.05
	p	1C-2; 1C-3		1C-2;1C-3
		2-3		2-3

Blood plasma diluted 200 fold
ABM concentration in the sample—19.6 nm/ml
Albumin auto-fluorescence excitation 286 nm
p-differences between groups are significant ($p>0.05$)

that the lipid compartment of the membrane interface became more rigid. Taken together, these findings reinforce our understanding that the cell membrane is a significant biological target of radiation. Thus the role of the biological membrane in the expression and course of cell damage after radiation exposure must be considered.

Our data agree with of Verma and Sonwalkar [15], who showed with Raman spectroscopy that, after x-radiation exposure, alterations occur in the plasma membrane that affect the state of the lipid-protein phase, the secondary structure of proteins, and the environment of aromatic amino acids. Protein–lipid interactions that are known to occur in specific domains with in the membrane may play an important role in mediating such radiation effects and experimental conditions associated with the development of oxidizing stimuli [1–3]. Changes in fluidity may influence the stability and accessibility of protein as well as its ability to associate or dissociate. The relevance of membrane fluidity as a physiological modulator of cellular functions nonetheless remains controversial.

Changes (alterations) in membrane proteins and lipids are accompanied by perturbations of binding site polarity [1].

Comparison of the maxima of the fluorescence spectrum of protein-bound and lipid-bound probe with that of the probe in different organic solvents [17] can provide information on binding sites. The fluorescence of ABM is sensitive to polarity changes in the microenvironment. A maximum max shift on passing from benzene to ethanol for an ABM is 67 nm [6, 7, 14]. Our results indicated that the probe ABM was localized in different transverse regions of the PBMC membrane for the groups of Chernobyl clean-up workers:

Group 1 emission wavelength maximum of ABM in the cell membrane is intermediate between that of ABM in benzene (583 nm) and in chloroform (625 nm). The position of emission maximum indicates that the environment of ABM in membrane is distinctly non-polar.

Group 2 emission wavelength maximum indicate that the environment of ABM in the membrane covers a wide region from non-polar to polar.

Taken together, the groups of clean-up workers differed between themselves in all tested parameters.

The results for groups tested suggest that various qualitative changes of membrane properties are evident in Chernobyl clean-up workers examined in 2006–2007 as compared with membrane characteristics joining examination in 1997. The results 1997 showed ABM fluorescence anisotropy values in Group 1 (A=0.261) and Group 2 (A= 0.245) higher than in 2007 (Group 1-A=0.233, Group 2 A= 0.219). In dynamics the membrane lipophilic apartment fluidity are increased in both groups of patients.

According to the obtained results (ANS anisotropy values, ABM binding with blood plasma albumin, albumin auto-fluorescence characteristics) (see Tables 1 and 2) that alterations in proteins is more remarkable in Group 1 as compared with Group 2.

Whereas on the contrary ABM fluorescence anisotropy values and spectral characteristics demonstrate (Tables 1 and 2) more pronounced changes of lipids in Group 2 as compared with Group 1. It can be concluded that the phospholipid compartment of cell membrane of Group 2 patients is more affected in comparison with the spectra obtained in healthy donors and Group 1. In our previous experiments (using probe 3-isopropyloxy-6-morpholino-2-phenilphenalen-1-ona) was obtained that in chronic B-cell lymphoid leukemia patients the fluorescence zone is also shifted to the long-wave region of spectrum and posses distinctively lower anisotropy values than those of healthy donors and chronic myeloleukemia patients [13].

The ABM and ANS spectral characteristics, fluorescence anisotropy values and albumin auto-fluorescence data (Tables 1 and 2) demonstrate in Group 1 the most pronounced alterations of proteins as compared with Group 2.

So ten years dynamic of PBMC membrane characteristics by ABM (spectral shift and anisotropy indexes) in Chernobyl clean-up workers reveal progressive trend toward certain resemblance with those of chronic B-cell lymphoid leukemia [13].

The principal cause of the increase of membrane damage was probably the long-live radioisotopes incorporated in the clean-up workers bodies as a permanent radiation source [18].

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