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Fluorescent Characteristics of Blood Leukocytes of Patients with Malignant and Nonmalignant Diseases

R. Brüvere,[1] N. Gabruševa,[1] I. Kalniņa,[2] G. Feldmane,[3] and I. Meirovics[2]

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Properties of the naphthalic acid derivative 3-isopropyloxy-6-morpholino-2-phenilphenalen-1-ona (PMPH) as a potential fluorescent probe were investigated. Spectral characteristics of the compound in different solvents as well as their binding to model lipid membranes (liposomes) and human peripheral blood mononuclear cells (lymphocytes) were determined. The fluorescence was found to be sensitive to polarity changes in the environment. Distinctions were observed in the spectral characteristics of the investigated compound when bound to liposomes and peripheral blood mononuclear cells of healthy donors, and patients with malignant and nonmalignant diseases. It was established that spectral characteristics of PMPH in cell suspension qualitatively characterize its structural and functional alterations during pathologic phenomena and correlate directly with the clinical view of disease. PMPH is shown to be a prospective method for screening for various pathologies.

KEY WORDS: Fluorescent probe; lymphocytes; diagnostics.

INTRODUCTION

Cellular mechanisms that are involved in malignant transformation of normal cells are associated with structural, functional, and dynamic changes in the cell surface membrane [1]. These changes are the principal determinants of the alterations of membrane fluidity observed in many human diseases. Recent studies of structure and function relationships in biological membranes have shown that membrane lipids play an important role in the regulation of cellular function. Many immunological functions may be heavily dependent on cell membrane structure [2].

[1] Biomedical Research and Study Centre, University of Latvia, Riga LV-1067, Latvia.
[2] Dep. of Organic Chemistry, Riga Technical University, Riga LV-1048, Latvia.
[3] Institute of Microbiology and Virology Medical Academy of Latvia, Riga LV-1067, Latvia.

It is very important for clinics to receive information on the properties of immune competent cells by an express method. The fluorescent probe proved to be an excellent, independent model for such studies [3]. This work investigated the possibility of using the fluorescent probe PMPH for detection of structural and functional alterations of cells. Such an analysis will have a great potential for determining the control mechanisms associated with the induction and development of the malignant transformed state in the hematopoietic system as well as in other mammalian tissue.

MATERIALS AND METHODS

The Fluorescent Probe

The fluorescent probe made from naphthalic acid derivative 3-isopropyloxy-6-morpholino-2-phenilphenalen -1-ona (PMPH) was synthesized at the Technical Uni-

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versity of Riga, Department of Organic Chemistry, Latvia [4]. Figure 1 shows the chemical structure of the probe PMPH.

Organic Solvents

Hexane, chloroform, and other agents were used as solvents for spectral and luminescence studies.

Liposomes

Liposomes were prepared from phosphatidyl choline using the method described in reference [5]. Liposomes were suspended in isotonic phosphate (pH 7.0) buffer (concentration, 0.5 mg/mL).

Study Subjects

The study subjects were (1) healthy donors ($n = 30$); (2) chronic myeloleukemia patients ($n = 28$); and (3) chronic B cell lymphoid leukemia patients ($n = 32$). The mean age was 51.6 ± 0.8 years.

Blood Collection

For each individual, peripheral blood (PB) samples were collected from a vein into disposable vacuum tubes containing preservative-free heparin 30 IU/mL for PB leukocyte (PBL) studies.

Preparation of PB Mononuclear Cells

The cells were isolated from freshly withdrawn heparinized venous blood by standard verographine method. Three milliliters of verographine was layered on 3 mL of blood and centrifuged at 2500 rpm for 30 min (60% verographine Spofa: aqua pro inj., 2:5). The ring of cells formed on the density interface was aspirated and washed three times with phosphate-bothered saline (PBS) (pH 7.3, osmolarity 290 mOsm) by centrifugation at 1500 rpm for 10 min and resuspended in RPMI 1640. The resulting cells suspension of 0.5×10^6 cells/mL was applied for the fluorescence measurements.

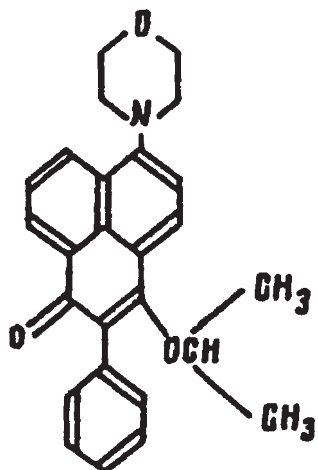

Fig. 1. Chemical structure of the PMPH probe.

Sample Preparation and Fluorescence Measurements

Investigations were performed by means of the newly synthesised fluorescent probe PMPH. The fluorescent dye mixture was dissolved in ethanol, resulting in an ethanol concentration of 0.1% in the probe. The blank sample for each experiment was prepared by PMPH titration without cells. The cell suspension was incubated with PMPH, resulting in a concentration of 18.5 μmol/L, at room temperature for 2 min. The time interval between the cell isolation and the fluorescence measurement was constant (3 hr) for all samples. Fluorescence parameters were registered on a Signe 4M (Latvia) spectrofluorimeter at excitation (λ_{ex}) wavelength of 250–500 nm and emission (λ_{em}) wavelength of 300–700 nm. Fluorescence intensity was measured in arbitrary units (F, a.u.).

Fluorescence Polarization

The method is based on analysis of the fluorescence polarization of the fluorescent probe, 1,6-diphenil-1,3,5-hexatriene (DPH) when embedded in the lipid core of the surface membrane of cells [3].

Labeling of cells with DPH was performed as follows: 0.1 mL of 2×10^{-3} M DPH in 2×10^{-3} tetrahydrofuran was injected into vigorously stirred 100 mL of 0.15 M KCI. Stirring was continued for 15 min at 25°C, and a clear dispersion of 2×10^{-6} M DPH, which is practically 2×10^{-6} void of fluorescence, was obtained. One volume of cell suspension (5×10^6–2.5×10^7 cells/mL) was mixed with one volume of the DPH dispersion. Upon penetration of DPH into the surface membrane, the signal increases steeply and levels off after about 60 min of incubation at 25°C. The labeled cells were then washed twice, resuspended in 0.15 M KCl, and immediately used for fluorescence measurements (λ_{em} 366 nm). Suspensions of unlabeled cells were used as reference samples.

The degree of fluorescence polarization (P) was calculated according to the equation

$$P = \frac{F_{11} - F_{\perp}}{F_{11} - 2F_{\perp}}$$

Table I. Spectral Characteristics (λ_{ex} 480 nm) of the Naphthalic Acid Derivative PMPH in Different Organic Solvents

Organic solvent	f	λ_{abs}nm	$\lambda_{ex,}$ nm	Quantum yield
Hexane	0.0011	562	574	0.54
Chloroform	0.0014	564	577	0.53
Benzene	0.0016	566	580	0.52
Ethylether	0.16	569	586	0.47
Tetrahydrofuran	0.2096	572	590	0.44
N, N′-dimethyl-formamide	0.2744	576	615	0.42
Acetone	0.2841	578	605	0.39
Ethanol	0.2887	584	615	0.36
Methanol	0.3098	585	619	0.22

Concentration of probe in organic solvents was 25 μM.

where F is fluorescence intensity parallel (F_{11}) and perpendicular ($F_{\perp}$) to the light polarization [6].

Statistical Analysis

Statistical differences and correlation of independent variables were determined using programs for the Student's test and Whitney-Mann *U*-test [7,8].

RESULTS

Spectral Characteristics of PMPH in Solvents

Spectral characteristics of compound PMPH dissolved in various organic solvents are shown in Table I. Solvents are listed in order of orientation polarity increase defined by the dielectric constant of the solvent and the refraction coefficient [3,9].

These results indicate that the fluorescence of this compound is sensitive to polarity changes in the microenvironment. The absorption spectrum is sensitive to the environment: The maximum wavelength of absorption spectrum for PMPH increases in correspondence with F. The fluorescence spectrum of probe in solutions strongly depends on the nature of the solvent, especially on its polarity (Table I). Maximum λ_{lm} change on passing from hexane to methanol for this probe is 45 nm.

Interaction of PMPH with Liposomes and Lymphocytes

Liposomes comprised of phosphatidyl choline alone (egg lecithin) and enriched with cholesterol showed different emission spectra maxima and fluorescence intensity (Table II). In comparison with phosphatidyl choline liposomes, a blue shift of emission spectra was noted in liposomes enriched with cholesterol (molar ratio, 2:1) and human peripheral blood lymphocytes (ly).

In chronic B-cell lymhoid leukemia patients' ly suspension, the PMPH fluorescence zone is shifted to the long-wave region of the spectrum (λ_{max} 615 nm). The maximum of the PMPH fluorescence spectrum in chronic myeloleukemia patients (λ_{max} 595 nm) was not changed compared with the data obtained from practically healthy donors (Fig. 2).

For the determination of membrane fluidity in liposomes, normal ly and observed patients' ly were first labeled with DPH and the degree of fluorescence polarization (P) was monitored (Table II).

The degree of fluorescence polarization of lecithin–cholesterol mixed liposomes was found to be much greater than that of the lecithin liposomes, thus indicating that

Table II. Spectral Characteristics and Degree of Fluorescence Polarization in Liposome and Lymphocyte Suspensions of Patients with Leukemic Diseases

Group under study	λ_{ex} (nm)	λ_{em} (nm)	F (rel.u.)	P
Liposomes (egg lecithin)	480	630	42.9	0.272 ÷ 0.287
Liposomes (egg lecithin + cholesterol)	480	650	18.6	0.325 ÷ 0.338
p_1			<0.001	<0.002
Lymphocytes (healthy donors)	480	595	20.3 ± 0.06	0.290 ÷ 0.296
Lymphocytes (chronic myeloleukemia patients)	480	595	22.7 ± 0.04	0.278 ÷ 0.291
p_1			>0.05	>0.05
Lymphocytes (B cell lymphoid leukemia patients)	480	615	41.8 ± 0.07	0.225 ÷ 0.254
p_1			<0.002	<0.001
p_2			<0.005	<0.002
Isotonic phosphate buffer	503	628	3.1	

p_1: Level of significance (between the leukemia patients and healthy donors); p_2: level of significance (between observed groups of patients).

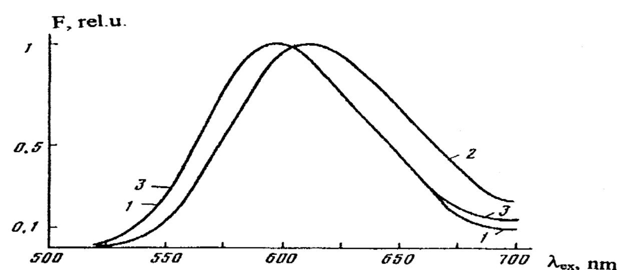

Fig. 2. Spectrum of the fluorescence emission of the PMPH probe (480 nm) in the lymphocyte suspension. (1) Healthy donors; (2) patients with chronic B cell lymphoid leukemia; and (3) patients with chronic myeloleukemia. Concentration of PMPH in the sample was 18.5 μM.

the lecithin liposomes are much more fluid as compared with the lecithin–cholesterol liposomes. It may be concluded, therefore, that increasing the value of cholesterol/phospholipids from 0 to 1 markedly decreases the fluidity of the lipid layers, as might be expected. The chronic B-cell lymphoid leukemia patients' ly tested possess distinctively lower P values than those of the healthy donors and chronic myeloleukemia patients' ly. No significant difference in results between healthy donors and chronic myeloleukemia patients was obtained.

The blue shift of emission spectra both in liposomes and ly occurs along with decreased fluorescence intensity F. PMPH fluorescence intensity in liposomes ($r = -879$) and cell suspensions ($r = -0.927$) is in inverse correlation with fluorescence polarization. When the fluorescence polarization properties of the fluorescence probe, DPH, were analyzed when embedded in membrane lipids, it was shown that there is a direct correlation between the degree of lipid fluidity in the cell surface membrane and the malignantly transformed state of cells from the hematopoietic system.

There is a good correlation between PMPH spectral characteristics, fluorescence polarization ($r = -0.927$), and clinical course if disease. In leukemia exacerbation, the obtained spectral parameters of ly were reduced more prominently (Table III).

DISCUSSION

PMPH spectral characteristics and membrane fluidity were different in observed groups of patients. The results might the explained by certain differences in properties of ly.

There are data about different histogenesis of lymphoproliferative diseases. The morphology of cells in chronic myeloleukemia patients does not significantly differ from the norm. Previous studies of other authors obtained morphological and immunological variety of ly in B-cell lymphoid leukemia patients. They present large amounts of pathological clones with lower stability of lipoprotein complexes [10–15]. These various types of structural alterations of tumor cells can be associated by functional activity and increase in fluidity of the surface membrane lipid layers [1,2].

A study of fluorescence polarization of human normal and leukemic ly failed to reveal any differences that could be attributed to the leukemic transformation.

Previous authors [1, 2] who studied with normal and leukemic populations from both experimental animals and humans have observed that the surface membranes of the leukemic cells have a more fluid lipid core than that of normal ly. The increase in fluidity in the leukemic cells is predominantly caused by a decrease of cholesterol/phospholipids in the cell surface membrane, and in human leukemia, it is in correlation with the acuteness of the disease.

Table III. PMPH Spectral Characteristics and Lymphocyte Blast Transformation Reaction (LBTR) Level of B-Cell Lymphoid Leukemia Patients

Group under study	Fluorescence intensity, F	Fluorescence polarization, P	LBTR% (phytohemagglutinin, 10 μg/mL)
Healthy donors	20.3 ± 0.06	0.290 ± 0.296	55.4 ± 0.4
First determined diagnosis	41.8 ± 0.07	0.225 ± 0.254	22.0 ± 0.7
p_1	<0.001	<0.001	<0.001
Remission	34.0 ± 0.08	0.259 ± 0.273	35.2 ± 0.6
p_1	<0.002	<0.05	<0.002
Exacerbation	44.1 ± 0.08	0.194 ± 0.231	20.3 ± 0.5
p_1	<0.001	<0.001	<0.001
p_2	<0.002	<0.05	<0.05

p_1: Level of significance (between the leukemia patients and healthy donors); p_2: level of significance (between remission and exacerbation phase of disease).

Moreover, in studies in which attempts have been made to elucidate the biological significance of membrane fluidity, it was shown that the reduction of membrane fluidity induced by the introduction of exogeneous cholesterol into the surface membrane of intact lymphoma cells from mice resulted in a marked inhibition of their tumorigenicity. Conversely, a controlled increase in membrane fluidity of normal ly induced by a reduction of membrane cholesterol resulted in a significant increase in the activation of the normal ly by plant mitogens [1,2].

A new fluorescent probe, a derivative of 3-aminobenzanthrone (ABM) [16,17] developed at the Riga Technical University was also used to characterize peripheral blood mononuclear cells of healthy donors, patients with several nonmalignant diseases (advanced lung tuberculosis [16,17], multiple sclerosis [18], and rheumatoid arthritis [19]), and of those who have been subjected to ionizing radiation during the clean-up work in Chernobyl [20].

Synthesis was performed by substituting the bromide atom in 3-bromobenzanthrone with an appropriate amine. Fluorescent microscopy revealed the distribution ABM in ly of cellular membranes (plasma, mitochondrial, and nuclear), but it was not found inside the nucleus [17].

Nonmalignant Diseases

The spectra and their wavelength maxima from healthy patients did not differ from those of patients with myeloleukemia or with nonmalignant diseases. Therefore the absence of blue-shift emission of spectra indicates no significant cholesterol increase in ly of observed groups of patients. These groups showed only differences in the fluorescence intensity *F* (Fig. 3). The *F* and functional activity in tuberculosis patients was found to depend on the nature and dynamics of the tuberculosis process and the predominance of the oxidative or productive inflammation phase [17]. The above-mentioned parameters also changed according to the phase (exacerbation or remission) and type (remitting or chronic progressive) of multiple sclerosis [18] and seropositive or seronegative form of rheumatoid arthritis [19].

The λ_{max} of ABM in the membrane is at 630 nm, which corresponds closely to that of the probe in chloroform. Therefore, the environment of the probe is nonpolar.

Chernobyl Clean-Up Workers

The screening of Chernobyl clean-up workers from Latvia showed five patterns of ABM fluorescence spectra (Fig. 4; Table IV).

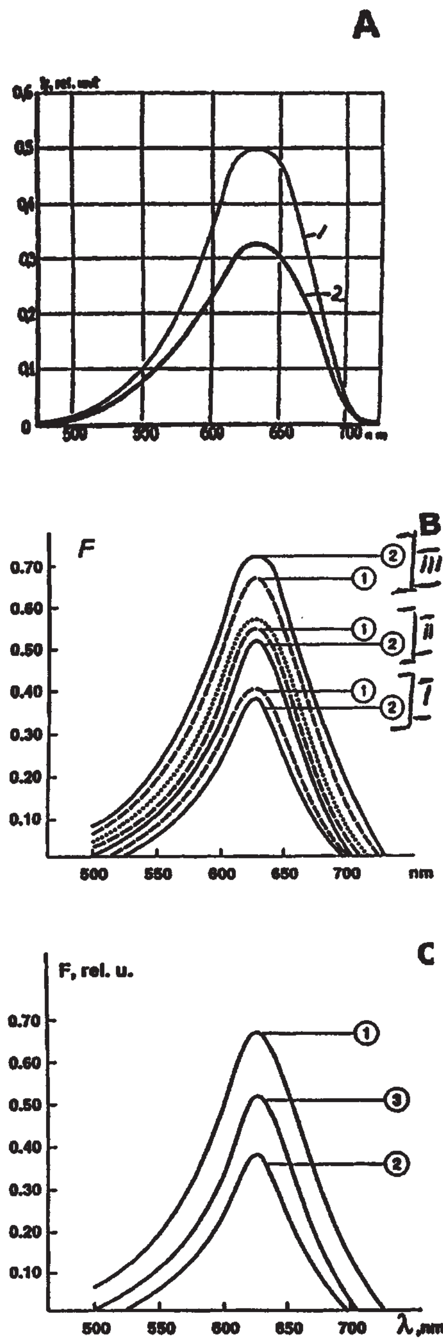

Fig. 3. Fluorescence emission spectra (λ_{ex} 470 nm) of ABM in peripheral lymphocyte suspension of healthy donors and patients with non malignant diseases. (A) Advanced lung tuberculosis [17]: (1) healthy donors; and (2) patients. (B) Multiple sclerosis [18]: Patients with (I) decreased fluorescence intensity (<0.38 a.u.); (II) normal *F* (0.47 ± 0.06 a.u.); and (III) increased *F* (>0.60 a.u.). Exacerbation (1) or remission (2) phase of disease. Dotted lines, healthy donors. (C) Rheumatoid arthritis (RA) [19]: (1) healthy donors; (2) patients with seronegative RA; and (3) Patients with seropositive RA. ABM concentration used in samples was 19.6 μM.

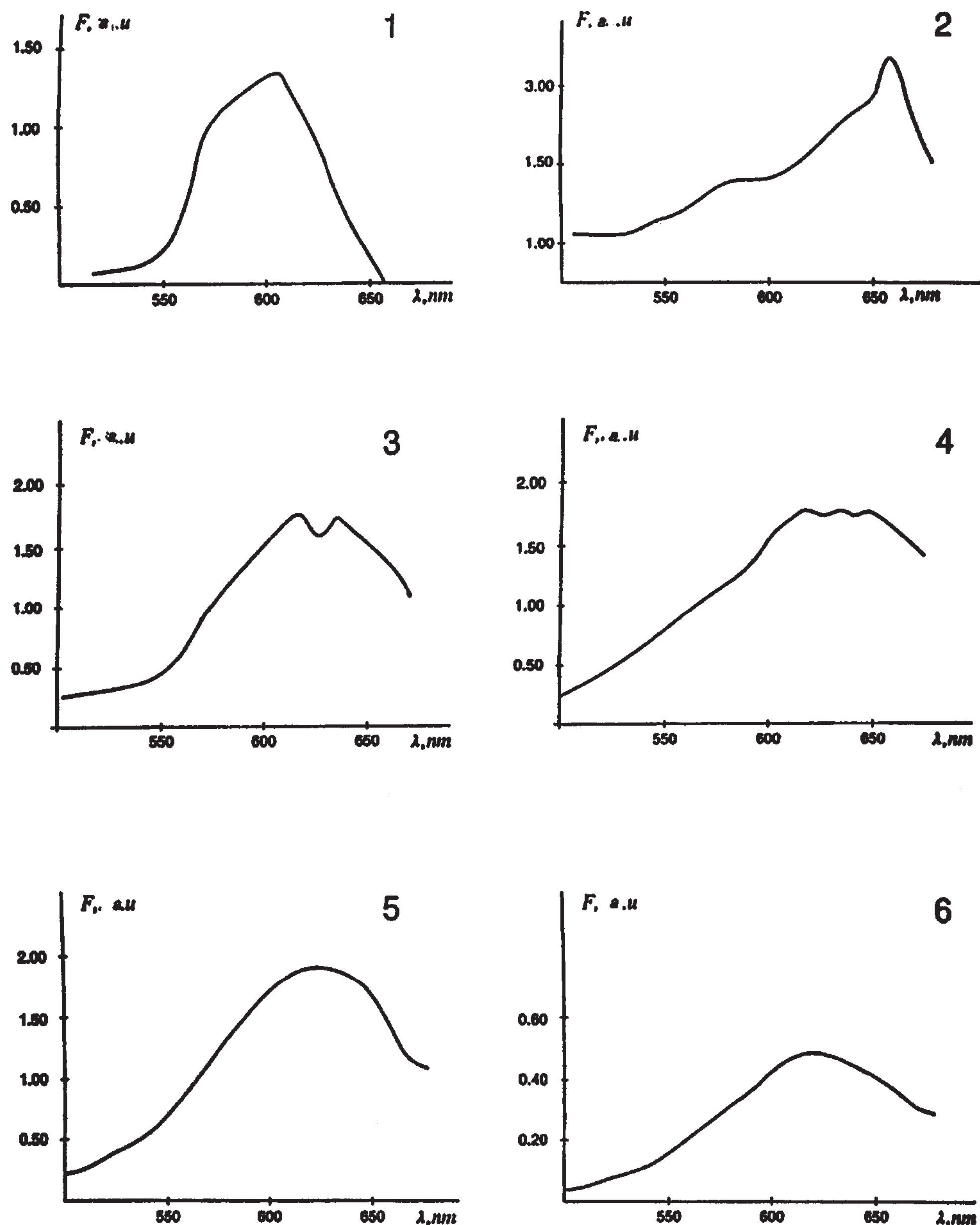

Fig. 4. Patterns of ABM fluorescence spectra in peripheral blood mononuclear cells of Chernobyl clean-up workers [20]. (1) The fluorescence zone is shifted to the short-wave region of spectrum by 3–13 nm (λ_{max} 517–627 nm). (2) The fluorescence zone is shifted to the long-wave region of the spectrum by 19–25 nm (λ_{max} 649–655 nm). (3) There are two bands fluorescence, with maxima at (λ619–629 nm) and (λ 645–655 nm). (4) A wide fluorescence maximum (plateau) is observed in the region of (λ 625–655 nm). (5) A fluorescence maximum at λ 630 nm corresponds to the fluorescence observed in healthy persons.

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The five groups of Chernobyl clean-up workers differed among themselves in intensity of fluorescence (F)

Table IV. Fluorescence Intensity of the Probe ABM in Peripheral Blood Mononuclear Cell (PBMC) Suspension and Several Parameters of Immune State of Chernobyl Clean-Up Workers [20]

				Interferons induced by				
Group no.	Groups of patients	F (a.u.)	PBMC 10^6 cells	NDV (IU)	dsRNS (IU)	PHA, (IU)	Age (years)	P
1	λ_{max} 617 ÷ 627 nm (n = 12)	1.49 + 0.10	6.8×10^9 $\pm 0.5 \times 10^9$	177.40 ± 37.52	22.70 ± 6.23	154.30 ± 36.14	41.75 ± 2.46	0.261 ± 0.025
2	λ_{max} 649 ÷ 655 nm (n = 14)	3.15 ± 0.15	6.8×10^9 $\pm 0.4 \times 10^9$	99.12 ± 21.25	95.36 ± 11.03	104.05 ± 10.93	45.23 ± 1.83	0.118 ± 0.002
3	λ_{max} 619 ÷ 629 nm λ_{max} 645 ÷ 655 nm (n = 37)	1.66 ± 0.08	7.1×10^9 $\pm 0.3 \times 10^9$	79.29 ± 8.74	65.95 ± 10.83	77.95 ± 12.29	40.10 ± 1.09	0.246 ± 0.004
4	λ_{max} 625 ÷ 655 nm (n = 17)	1.67 ± 0.07	7.1×10^9 $\pm 0.4 \times 10^9$	84.05 ± 23.19	28.31 ± 11.07	80.84 ± 28.04	41.19 ± 1.70	0.245 ± 0.004
5	λ_{max} 630 nm (n = 17)	1.79 ± 0.07	7.2×10^9 $\pm 0.5 \times 10^9$	116.12 ± 18.44	70.02 ± 16.48	73.64 ± 16.52	41.56 ± 1.58	0.236 ± 0.003
	P [a]	1–2 1–5 2–3 2–4 2–5		1–2 1–3 1–4 1–5	1–2 1–3 1–5 2–4	1–3 1–4 1–5 2–3		1–2 1–3 1–1 1–5 2–3 2–4 2–5 3–5 4–5

F: Fluorescence intensity; P: fluorescence polarization; NDV: Newcastle disease virus; PHA: phytohemagglutinin; dsRNA: double stranded ribonucleic acid.
[a] Differences between groups are significant, $P < 0.05$.

and anisotropy index (P): F was significantly higher and P was lower in group 2 in comparison with the others, and group 1 differed from group 5. The lowest F in clean-up workers was more than three times higher than that previously observed in healthy donors. When the observed groups were combined, a positive correlation between F and λ_{max} ($r = +0.774$) and a negative correlation between F and fluorescence polarization ($r = -0.974$) were observed (Table IV).

Yonei and Kato [21] showed that changes in the fluidity of irradiated membranes primarily were due to conformational and biochemical changes of membrane proteins and/or lipids.

The effects of ionizing radiation on biological membranes include alterations in membrane proteins and peroxidation of unsaturated lipids accompanied by perturbations in the lipid bilayer polarity. These changes are associated with the destruction of the cell membrane surface through conformational alterations of proteins [22].

Changes of membrane structure obviously influence the incorporation of ABM into the cell membrane [16–20]. The spectral characteristics obtained in cells of Chernobyl clean-up workers (five spectral patterns) are due to ABM fluorescence originating from lipid-bound ABM and from protein-bound ABM.

Slavik [9] found that damage to membrane proteins or lipids could not be directly detected by fluorescent probe spectral characteristics. We cannot also exclude the fact that protein alterations influence the structure and dynamics of the surrounding lipids.

Comparision of the maxima of the fluorescence spectrum of a protein-bound and a lipid-bound probe with that of the probe in different organic solvents [23] indicated that the probe ABM was localized in different transverse regions of the ly membrane for the five groups of Chernobyl clean-up workers from distinctly nonpolar (group 1) to quite polar (group 4).

Four of the obtained fluorescence patterns (groups 1–4) had never been seen in healthy individuals (who had had no professional contact with radioactivity) or patients with nonmalignant diseases.

Obtained patterns of spectra suggest that specific and qualitatively different changes of membrane properties are evident in Chernobyl clean-up workers' cells, similar to that in lymphoid leukemia patients. In all observed groups of patients with nonmalignant and malignant diseases and Chernobyl clean-up workers, correlation was

obtained between probe fluorescence intensity, anisotropy, λ_{max}, and the ability of cells to produce interferons when induced in vitro by Newcastle disease virus of phytohemagglutinin. The spectral characteristics of probes and functional activity of cells correlate with clinical and laboratory characteristics of the groups.

Fluorescent probes PMPH and ABM offer a prospective method for screening and functional alterations of cell membranes for various pathologies.

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