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SHORT COMMUNICATION

Fluorescent Probe ABM for Screening Gastrointestinal Patient's Immune State

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Abstract The fluorescent probe-aminoderivative of benzanthrone, ABM (developed at Riga Technical University, Riga, Latvia) was used to characterize the membranes of lymphocytes of cancer patients: 46 patients with gastrointestinal diseases, 13 patients having different primary localizations with massive metastases and intoxication. Patients were divided into three groups: (1) with decreased fluorescence intensity, (2) normal fluorescence intensity, (3) increased fluorescence intensity. The lymphocytes distribution among subsets differed between groups, in correspondence to the level of florescence intensity. Surgical treatment affected the main immunological parameters and elevated the functional activity of lymphocytes. In the advanced tumors group, fluorescence intensity correlates with the survival rate. Results suggest that determination of lymphocytes functional activity by ABM can aid evaluation of the immune status in cancer patients.

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Abbreviations

ABM	a derivative of 3-aminobenzanthrone (conditional name)
r	correlation coefficient

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 and important role in the regulation of cellular function. Many immunological functions may be heavily dependent on cell membrane structure [2–5].

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 [6]. This work investigated the possibility of using the fluorescent probe ABM for detection of structural and functional alterations of cells. Such an analysis will have a great potential for determination the control mechanisms associated with the induction and development of the malignant transformed state in the hematopoietic system as well as in the mammalian tissue.

In our work we determined the fluorescence intensity of ABM in suspensions of peripheral blood lymphocytes from cancer patients, in relation to other immunological

parameters. Our aim was to elaborate a new fluorescent-based method for immune diagnostics of gastrointestinal cancer patients.

Materials and methods

Study subjects Lymphocytes spectral characteristics and their distribution into subsets in a large group of 46 patients suffering from gastrointestinal cancer were determined. Fourteen healthy volunteers were also observed to determine the normal level of lymphocytes fluorescence intensity and other characteristics.

For more detailed analysis, we selected ten gastric cancer patients in stage III and 10 colorectal cancer patients in stages II–III, who showed no signs of other diseases and postoperative complications. These patients were tested 1 day before operation and 10 days after surgical treatment. Mean values for the whole gastrointestinal cancer group was calculated, combining the gastric cancer and colorectal cancer groups.

Thirteen patients with different primary localizations of cancer, with massive cancer metastases and intoxication were observed. These patients had received symptomatic treatment in the Department of Palliative and Supportive Care.

Blood collection Blood was drawn from the patients (cancer patients and healthy volunteers) at 8–10 A.M. Peripheral blood samples were collected from vein into vacuum tubes containing preservative-free heparin 30 IU per tube (7 ml).

Preparation of lymphocytes Cells were isolated from freshly withdrawn heparinised venous blood by standard verographine method. Three ml of verographine was layered on 3 ml of blood and centrifuged at 1,700×*g* for 30 min (60% verographine). The ring of cells formed on the density interface was aspirated and washed three times with sodium–potassium buffered saline (pH 7.3) by centrifugation at 1,700×*g* for 10 min, and re-suspended in RPMI 1640. The resulting lymphocytes suspension containing 5×10^6 cells/ml was used for the fluorescence measurements.

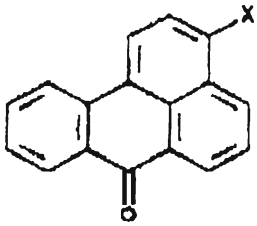
Sample preparation and fluorescence measurements Investigations were performed by means of the newly synthesized fluorescent probe ABM [7–9]. The cell suspension without probe was used as a "blank" sample for each experiment. The ABM was dissolved in ethanol in a concentration of 19.6 μmol/l. 1 ml of cell suspension was incubated with 0.01 ml ABM ethanol solution at room temperature for 2 min. The time interval (3 h) between cells isolation and the fluorescence measurement was constant for all samples. Fluorescence parameters were registered on a "Signe4M" (Latvia) spectrofluorimeter at excitation wavelength of 470 nm and emission wavelength of 520–700 nm. To register the luminescence from the surface of its excitation, the sample was placed into a $1\times10\times40$ mm^3 cuvette fixed at an angle of 30° to the excitation light beam. Fluorescence intensity was measured in arbitrary units (*F*, a.u.).

Determination of lymphocyte subsets We have determined the absolute number of lymphocytes in 1 ml of blood, and also the relative number of these cells by routine blood analysis. Determination of lymphocyte subsets was done with Ortho Spectrum flow cytometer. We estimated the relative numbers of lymphocytes subsets and calculated also their absolute numbers in 10.9 cells/l of blood.

Statistical analysis Statistical differences among groups having different spectral characteristics were determined using the Student's *t* test and Whitney–Mann's *U* test. Correlative relationships between spectral characteristics of ABM and parameters of the immune state were determined [10].

Results

ABM: distribution and spectral characteristics in cells A new fluorescent probe, a derivative of 3-aminobenzanthrone (ABM) [7, 8] developed at the Riga Technical University. Synthesis was performed by means of substituting the bromine atom in 3-bromobenzanthrone with an appropriate amine.



The chemical structure of the ABM bears a resemblance to the structure of the fluorescent probe MBA (3-methoxybenzanthrone), having substituted aminogroup at 3 position of benzanthrone molecule. Despite its extensive spectrum of possibilities, the use of MBA is limited by two properties of this probe: first, it destroys cells after a short period of time; and fades rapidly in fluorescent light (approximately 80% in 6–8 min). Our data witness that ABM is photostable and nontoxic for cells.

Fluorescent microscopy revealed the distribution of ABM in such cell membranes as plasma, mitochondrial,

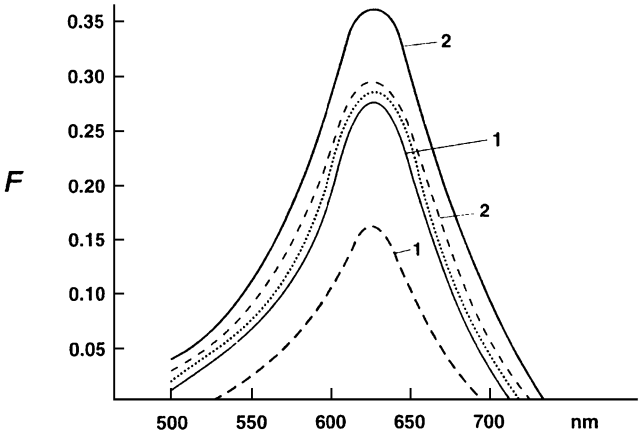


Fig. 1 Fluorescence emission spectra (excitation wavelength, 470 nm) of ABM in human peripheral blood lymphocyte suspensions before (*1*) and after (*2*) surgical treatment. *Full lines* colorectal cancer patients; *dashed lines* gastric cancer patients; *dotted lines* healthy donors. ABM concentration in the sample was 19.6 nM

and nuclear, but there were no signs of localization of probe inside the nucleus. The environment of the fluorescent probe is quite polar, similar to that of methanol [7, 8]. The addition of Triton X-100 did not lead to any changes in ABM fluorescence intensity, while incubation of lymphocytes at 37 C resulted in increased ABM fluorescence intensity. The above facts suggest localization of ABM deep in the phospholipids bilayer [7, 8].

In the previous experiments fluorescent probe ABM was used to characterize peripheral blood mononuclear cells of healthy donors, patients with several nonmalignant diseases (advanced lung tuberculosis [7, 8], multiple sclerosis [11], and rheumatoid arthritis [12], and of those who have been subjected to ionizing radiation during the clean-up work in Chernobyl [13, 14].

For the better understanding obtained results and its interpretation it is necessary to remember the results of these investigations.

1. Microviscosity. ABM fluorescence intensity in lymphocytes suspension inversely correlated with membrane microviscosity (fatty acid composition).
2. Functional activity. Changes in membrane of cells have been shown to correlate not only physical and chemical changes of the membranes, but also with decline of cells responsiveness to mitogens (functional activity). We measured spectral characteristics of native and mitogen-stimulated lymphocytes taken from healthy test donors. Isolated cells were stimulated in vitro by mitogens, such as phytohemagglutinin (PHA-P) and concanavalin A (Con A) (mostly T-cells) and poke weed mitogen PWM (mostly dependent B cells). At the same time, characteristics of the lymphocytes blast transformation reaction (LBTR) (DNA synthesis activity) in these lymphocytes were measured. It was ascertained that the method is more sensitive when using PWM at a lower concentration of ABM as with PHA and Con A. Results testify that spectral parameters of ABM in the cell suspension depend on the mitogen used, its dose, and on the subpopulation of lymphocytes stimulated. ABM is therefore prospective for the determination of the proliferative activity of lymphocytes [7, 8].
3. Phenotypical characteristics. Lymphocytes distribution among detected subsets (relative and absolute numbers) had differences in observed patients groups corresponding to a certain level of fluorescence intensity (decreased, normal, and increased). ABM fluorescence intensity in lymphocytes suspension qualitatively characterizes alterations of the organism immune state, correlates directly with the clinical view of the disease and has specific differences according to its phase and type [11–14].

It can be concluded that in all investigated pathologies spectral parameters of ABM in cell suspension reflects several interdependent (interrelated) properties of lymphocytes: (1) physicochemical state of their membrane, (2) membrane microviscosity, (3) proliferating activity of lymphocytes, (4) lipid metabolic activity, (5) phenotypical characteristics of lymphocytes. The observed changes of the studied parameters reflect alterations of the cellular mechanisms of immunity and therefore can be applied as prelim-

Table 1 ABM fluorescence intensity in cell suspension and peripheral blood lymphocyte subpopulations count in gastrointestinal cancer patients

Gastrointestinal cancer (colorectal II+III stages+gastric III stage cancer) (n=26)									
No.	*F*, a.u.	Ly (%)	$CD3^+$ absolute number	$CD4^+$ absolute number	$CD8^+$ absolute number	$CD16^+$ (%)	$CD16^+$ absolute number	$CD38^+$ (%)	$CD4^+/CD8^+$
1[a]	0.20± 0.03	26.80± 2.01	1,116.21± 119.20	697.87±73.53	585.40±72.81	13.48± 30.1	280.41±30.12	5.04± 0.97	1.36± 0.07
2[b]	0.31± 0.04	20.33± 1.47	1,063.77± 113.62	613.64±61.08	450.93±58.01	7.76± 1.09	132.14±21.08	10.68± 2.06	1.55± 0.10

Mean value of the fluorescence intensity in the control group is 0.25±0.06 a.u.
[a] Before surgical treatment
[b] After surgical treatment

Table 2 ABM fluorescence intensity in cell suspension and peripheral blood lymphocyte subpopulations count in gastrointestinal cancer patients

Colorectal cancer (II–III stages) (n=10)				Gastric cancer (III stages) (n=10)		
No.	F, a.u.	$CD16^+$ (%)	$CD16^+$, absolute number	$CD4^+/CD8^+$	F, a.u.	Ly (±%)
1[a]	0.28±0.05	15.95±2.18	314.18±39.27	1.27±0.10	0.16±0.03	32.80±2.85
2[b]	0.36±0.05	7.93±1.43	144.75±22.44	1.55±0.13	0.29±0.06	21.96±1.60

Mean value of the fluorescence intensity in the control group is 0.25±0.06 a.u.
[a] Before surgical treatment
[b] After surgical treatment

inary screening test in immune diagnostics. There is a strong correlation among all studied parameters, clinical and laboratory characteristics of the patients groups. The fluorescence intensity and functional activity lymphocytes 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 [7, 8]. The abovementioned parameters also changed according the phase (exacerbation or remission) and type (remitting or chronic progressive) of multiple sclerosis [11] and seropositive or seronegative form of rheumatoid arthritis [12].

ABM binding with cell membrane In gastrointestinal cancer patients ABM emission spectra maximum was not changed in comparison with the same parameter in healthy control group (Fig. 1).

Before surgical treatment fluorescence intensity in the common gastrointestinal cancer group and colorectal cancer groups did not differ from the control mean value (0.25± 0.06 a.u.), but was decreased in gastric cancer patients (0.16±0.03 a.u.). After the operation fluorescence intensity increased in all observed groups by 55.0, 28.6 and 81.5%, respectively, and in colorectal cancer patients it was higher (0.36±0.05 a.u.) than in the control.

Tables 1 and 2 show the statistically significant differences in ABM fluorescence intensity not only among observed patients groups, but also before and after surgical treatment.

These results (different ABM spectral characteristics) can be explained by different membrane properties in observed groups of patients.

Lymphocyte count and subpopulations In the common gastrointestinal cancer group the absolute number of CD3+ killer cells, CD4+ helper cells, CD16+ natural killer cells and the relative number of CD16+ cells diminished (still the ration CD4+/CD8+ increased). In the colorectal cancer group the absolute and the relative number of CD16+ cells decreased, the CD4+/CD8+ ratio increased (Tables 1 and 2).

Before operation in the all patients groups fluorescence intensity correlated with the relative number of CD38+ cells (r=+0.958, +0.956 and +0.859, respectively), supporting the view that fluorescence intensity depends on the level of activated lymphocytes. After surgical treatment in common gastrointestinal cancer and colorectal cancer groups, a reverse correlation of ABM fluorescence intensity was found with respect to the relative count of CD16+ cells (r=−0.791 and r=−0.498, respectively), but in the gastric cancer group it was held with respect to the relative count of CD8+ cells (r=−0.804) (Tables 1 and 2).

Subpopulations of lymphocytes in relation to fluorescence level The 46 patients with gastrointestinal cancer or benign gastrointestinal diseases were divided into three groups: (1) group, with decreased fluorescence intensity, lower than 0.17 a. u.; (2) group, with normal fluorescence intensity (0.17–0.38 a. u.), and (3) group, with increased fluorescence intensity (higher than 0.38 a. u.).

The distribution of lymphocytes among the subpopulations both in relative (Table 3) and in absolute numbers

Table 3 Relative numbers of peripheral blood lymphocyte subpopulations (%)

Group no.	F, a.u.	Ly (%)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4^+/CD8^+$	$CD38^+$ (%)	$CD16^+$ (%)	$CD19^+$ (%)	$CD3^+$ (%)
1 (n=17)	0.13±0.02	33.7±2.7	28.6±3.2	26.1±2.0	1.18±0.12	15.3±0.8	11.8±1.2	4.3±0.6	51.7
2 (n=15)	0.27±0.02	27.0±1.3	38.4±2.1	19.5±2.1	1.88±0.16	17.6±1.6	12.5±1.1	4.9±0.8	51.1
3 (n=18)	0.56±0.02	23.5±2.2	40.7±2.6	23.4±1.8	1.74±0.15	24.0±1.7	13.9±1.3	7.3±0.8	53.7
	1–2	1–2	1–2	1–2	1–2	1–2	–	1–3	–
	1–3	1–3	1–3		1–3	1–3		2–3	
		2–3				2–3			

Table 4 Absolute numbers of peripheral blood lymphocyte subpopulations in 1 mm^3 or 10^9 cells/l of blood (%)

Group no.	*F*, a.u.	Ly	$CD4^+$	$CD8^+$	$CD38^+$	$CD16^+$	$CD19^+$	$CD3^+$
1 (n=17)	0.13±0.02	2.83±0.21	0.81±0.12	0.76±0.08	0.47±0.06	0.30±0.04	0.11±0.04	1.56±0.18
2 (n=15)	0.27±0.02	2.33±0.12	0.91±0.07	0.49±0.02	0.43±0.05	0.29±0.03	0.11±0.02	1.24±0.09
3 (n=18)	0.56±0.02	1.48±0.1	0.61±0.07	0.35±0.05	0.31±0.06	0.19±0.01	0.10±0.01	0.70±0.11
p<0.05	1–2	1–3	2–3	1–2	1–3	1–3	–	1–3
	1–3	2–3		1–3	2–3	2–3		2–3
				2–3				

(Table 4) differed significantly between the groups in relation to the certain level of fluorescence intensity.

Among these groups an inverse relationship between fluorescence intensity and the relative number of CD8+ cells (r=−0.743) and the direct relationship with the CD4+/CD8+ ratio (r=+0.796) was detected.

Patients receiving palliative and supportive care Survival rates after the first estimation of fluorescence intensity were 0–6 months for six patients (group 1) suffering from cancer of different primary localizations, with massive metastases and intoxications; and three patients lived 24 months or more (group 3). The fluorescence intensity levels were significantly higher in group 2 than in group 1 and in the control group. In this group of patients in contrast to other patient groups, we observed that the relative and absolute numbers of lymphocytes had direct (not inverse) correlations with fluorescence intensity (r=+0.696 and r=+0.704, respectively). Patients who lived more than 24 months had high levels of lymphocyte functional activity and also higher relative and absolute numbers of lymphocytes, in comparison with patients who lived only 0–6 months (Table 5).

In comparison with stage II local cancer, the functional activity of lymphocytes, estimated as ABM fluorescence intensity, tended to increase with progression of cancer.

Table 5 ABM fluorescence intensity in cell suspension peripheral blood lymphocyte count and survival rate in patients with advanced tumours

No.	Survival rate (months)	*F*, a.u.	Ly (%)	Ly, absolute number
1	0–6	0.25± 0.03	11.66± 1.38	1,091.15± 106.48
2	>24	0.53± 0.11	23.27± 5.59	1,388.98± 214.01
p<0.05 between groups	1–2	1–2	1–2	1–2

Discussion

ABM incorporates deeply into the phospholipids bilayer of the plasma membrane. The ABM fluorescence intensity in cells suspension changes according to its environment polarity and, consequently, in relation to the plasma membrane microviscosity. The microviscosity of the plasma membrane has been shown to correlate with lipid metabolic activity [1, 2]. There are various pathological states in which the lipid composition and content of fatty acid in the lymphocyte plasma membrane are disturbed [2]. It is known that various human cancers result in changes in the membrane composition of host tissues. For example, colorectal cancer patients have abnormal plasma and erythrocyte fatty acids, as well as their polyunsaturated metabolites [4].In lymphocytes, membrane-associated events, especially lipid metabolism, play a pivotal role in signal transduction, all of which are important in immune cell function [4].

ABM spectral characteristics (fluorescence intensity) were different in observed groups of gastrointestinal patients. This finding indicates a different physical (structural and functional alterations in the observed groups of patients. Increase of ABM fluorescence intensity after operation is due to compensatory rise of functional activity in response to a decrease in the count of the lymphocytes subsets. These findings depend on the different activation of each lymphocyte subsets during the period of surgical treatment (see Tables 1 and 2).

Results show that the relative numbers of lymphocytes of specific subsets correspond to particular levels of fluorescence intensity-decreased, normal or increased. This suggests that fluorescence intensity of different lymphocytes subpopulations is distinct and that each subset provides a specific contribution to the fluorescence intensity of the whole lymphocytes suspension. (Tables 3 and 4).

The inverse relationships of the relative and absolute numbers of lymphocytes to fluorescence intensity in patients divided into three groups according to the fluorescence intensity level (decreased , normal and increased), as well as the inverse correlations between lymphocyte numbers and fluorescence intensity, in gastric and colorectal cancer

groups suggest a compensatory reaction of rising lymphocytes functional activity (expressed as increasing fluorescence intensity) to a decrease of lymphocyte number.

Our observation that the relative number of CD4+ cells was lowest, and that the relative and absolute numbers of CD8+ cells were highest, in the group with decreased fluorescence intensity, were expected, because CD4+ helper cells stimulate and CD8+ (suppressor and cytotoxic) cells inhibit the immunological response, thereby affecting the functional activity of the whole lymphocytes suspension, estimated as fluorescence intensity.

The highest ratio of CD4+/CD8+ was in the group with increased fluorescence intensity and the lowest ratio in the group with decreased fluorescence intensity.

Also, as expected, the relative number of CD38+ cells increased with an increase of F in the cancer patient groups, grouped according to the fluorescence intensity level. The CD38+ cells mainly consist of activated T lymphocytes, and therefore, the functional activity of the lymphocyte population is greater if the number of CD38+ cells is high.

These results are in agreement with the correlations between CD38+ cells and fluorescence intensity observed in gastric and colorectal cancer groups as well as in the whole gastrointestinal cancer group before surgical treatment. After operation, these correlations disappeared, but both relative and absolute numbers of CD38+ cells increased in the colorectal cancer group. This may be due to the partial loss of functional activity of CD38+ cells, when simultaneously expressed CD38+ antigens stay and remain on the cell's surface.

The numbers of natural killer (CD16+) cells was not related with ABM fluorescence intensity F and the functional activity of the whole lymphocyte subpopulation.

We suppose that the significantly higher relative number of B lymphocytes (CD19+ cells) in the group with increased fluorescence intensity provides evidence of contribution of effector cells of humoral immunity in the common functional activity of the lymphocyte population.

A significantly lower absolute number of CD3+ cells in the group with increased functional activity of lymphocytes may indicate that in patients with a low number of T lymphocytes (CD3+ cells) their functional activity compensatingly increases.

Our results showed that fluorescence intensity values can be used to estimate the distribution of lymphocytes among different subsets and its functional activity. It is very important for clinical immunological screening instead of more expensive routine tests.

The results shown for the gastric cancer and colorectal cancer groups allow us to conclude that surgical treatment affects the immunological parameters and elevates the functional activity of lymphocytes.

Functional activity of lymphocytes, detected as fluorescence intensity, exhibits a tendency to increase with progression of cancer. It is possible, that high levels of lymphocytes functional activity have certain roles in the prolongation of survival rates in patients with disseminated cancer. We suggest that special stimulation of lymphocytes functional activity can exert favorable influence on patient's survival and life quality in late stages of the disease.

Spectral parameters of ABM reflect a wide range of interrelated (interdependent) characteristics of lymphocytes physicochemical state and microviscosity of membrane: proliferating and lipid metabolic activity of cells: phenotypical characteristics of lymphocytes (distribution of lymphocytes among subsets).

The observed changes of the studied spectral parameters are probably coupled with alterations of the cellular mechanisms of immunity regulation and therefore can be applied as screening test in immune diagnostics. The obtained results suggest that spectral parameters of ABM in cell suspension reflects alterations in the cellular mechanisms of immunity, therefore fluorescent method can be used as preliminary screening test in immune diagnostics.

The fluorescence-based method is less expensive and time consuming, technically simple, 100 times more sensitive than absorption methods.

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