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Fluorescent Characteristics of Rheumatoid Arthritis Patients Blood Lymphocytes

Inta Kalniņa,[1,4] Tija Zvagule,[2] Rūta Brūvere,[3] and Imants Meirovics[1]

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A fluorescent probe, ABM, aminoderivative of benzanthrone, synthesized in the Department of Organic Chemistry of the Riga Technical University (Latvia), has been successfully used to characterize changes in the structural and functional properties of cell membranes during different pathologies. In the present study the physicochemical properties and the functional activity of the peripheral blood mononuclear cells (lymphocytes—Ly) in patients with rheumatoid arthritis (RA) were studied using the ABM probe. Intensity of the ABM fluorescence in the celi suspension, functional activity of the ly anisotropy of the membranes differ patients with different titres of rheumatoid factor in blood. Patients with seropositive RA had decreased proliferative activity and lower number of iy in blood plasma indicating greater alterations of the immunoregulating processes in these patients as compared to patients with seronegative RA. In the latter the Ly deficiency is compensated to some extent by increased proliferation activity of these cells. The ABM fluorescence intensity correlated not only with membrane anisotropy ($r = 0.97$, but also with the proliferation activity of the Ly ($r = 0.98$). The above parameters correlated with the clinical manifestations of the disease. The results indicate that the fluorescent probe ABM is useful for screening the physicochemical status of Ly membranes and the proliferation activity of these cells in RA patients.

KEY WORDS: Fluorescent probe; lymphocytes; rheumatoid arthrilis; diagnostics.

INTRODUCTION

Development of different immunopathological reactions provoked by alterations of cellular and humoral immunity is one of the main stages of pathogenesis of rheumatoid arthritis (RA), a chronic systemic inflammation all disease of articulations [1]. According to pathological changes in the immune system the (RA) is classified as a deficiency of the T-suppressors. It is considered that a decrease in the number of T-lymphocytes is not the only alteration of the immune system in RA, as the functional activity of these cells, and regulation in the T-suppressor subpopulation and their interaction with other regulatory cells are also affected [2,3].

Further studies of the proliferation activity of Ly as one of the cellular components of the immune system in patients with RA, could aid to in elucidating several aspects of pathogenesis and development of this disease. The goal of this study was to determine the physicochemical status of the membranes and the proliferation activity of the peripheral blood Ly in patients with RA. Fluorescent probes have been successfully used for this purpose [4]. This study employed a novel fluorescent probe ABM (conventional designation) [5]. The observed changes in the

[1] Departmant of Organic Chemistry, Riga Technical University, Riga, Latvia.
[2] Outpatients Department, Paul Stradins State Clinical Hospital, Riga, Latvia.
[3] Biomedical Research and Study Center, University of Latvia, Riga, Latvia.
[4] To whom correspondence should be addressed. E-mail: lulc@lanet.lv

ABBREVIATIONS: ABM, A derivative of 3-bromobenzanthrone (conditional name); F, Fluorescence intensity; LBTR, Lymphocyte blast transformation reaction; 1y, Lymphocyte; PBMC, Peripheral blood mononuclear cells; PHA, Phytohemagglutinin; R, Correlation coefficient; *r*, Anisotropy; RA, Rheumatoid arthritis; RF, Rheumatoid factor.

there fluorescence properties of this probe incorporated in the membranes of Ly were compared to the degree of the disease.

MATERIALS AND METHODS

Study Subjects

Eighty-five patients with classical RA were observed. The group consisted of 65 women and 20 men (age 22–60 years, duration of the disease 2–25 years). It is well known that the rheumatoid factor (RF) of the M class of immunoglobulins is an important feature of the pathological process during the RA and is of diagnostic importance. According to the concentration of RF in blood, the patients were divided into two groups: 49 patients with seronegative form of the RA, 36 patients with the seropositive RA form.

Blood Collection

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

The Fluorescent Probe ABM

A new fluorescent probe, aminoderivative of benzantrone (ABM) [5] developed at the Riga Technical University (Latvia) was used in this work. Fluorescent microscopy revealed the distribution ABM in cellular membranes of Ly (plasma, mitochondrial and nuclear), but was not found inside the nucleous [5].

PBMC Preparation

Mononuclear cells (PBMC) were isolated from freshly withdrawn heparinised venous blood by standard Verographine method. Three milliliters of Verographine were layered on 3 mL of blood and centrifuged at 2500 *g* for 30 min. (60% Verographine Spofa: aqua pro inj., 2:5). The band of PBMC formed on the density interface was aspirated and washed three times with PBS (pH 7.3 Osmolarity was 290 mosmol) by centrifugation at 1500 *g* for 10 min., and resuspended in RPMI 1640. The resulting PBMC suspension containing 0.5×10^6 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 [6,7]. The fluorescent dye ABM was dissolved in ethanol. The "blank" sample for each experiment was prepared by ABM titration without cells. The PBMC suspension was incubated with ABM at a concentration of 19.6 μmol/L, at room temperature (18–20°C) for 2 min.

The time interval (3 h) between cell isolation and the measurement of fluorescence was constant for all samples. Fluorescence on a "Signe 4M" (Latvia) spectrofluorimeter at excitation (λ_{ex}) wavelength of 470 nm and emission (λ_{em}) wavelength of 520–700 nm. Fluorescence intensity was measured in arbitrary units (F, au). Illumination—xenon lamp DksSh 500. Temp of measurements—1 s, monochromators slits—2.86 nm.

The amount of the ABM bound probe (expressed in μmol per l mL of ABM suspension) was determined as the difference between the total amount of the probe added to 1 mL of PBMC suspension and the amount of free probe. The content of the free probe in the supernatant was determined using the calibration curve (fluorescence intensity plotted versus the concentration of the probe added to the supernatant).

Membrane Anisotropy

Membrane anisotropy "r" was calculated according to the equation

$$r = \frac{F_{11} - F_1}{F_{11} + 2F_1}$$

where F is fluorescence intensity parallel (F_{11}) and perpendicular (F_1) to the light polarization [4].

Proliferation Activity of Peripheral Blood Leukocyte

The proliferation activity of the cells was determined by the DNA synthesis (radioisotope method).

For Ly blast transformation reaction (LBTR), celis were resuspended in RPMI 1640 with 25 mM glutaminc, penicilin (100 U/mL), streptomycin (100 mg/mL), and 10% of p-(IV)-inactivated serum. Triplicate cultures of 2×10^5 healthy PBMC were set up in microtitre wells containing 200 mL of pure culture medium (K) or modified with phytohemagglutinin (PHA-P, Serva, Germany—1, 5, 10 μg/well): and incubated 96 h at 37°C in 5% CO_2. To measure the proliferative activity of ly, 1 μCi of ^{3}H-thymidine (spec. act. 8,2 Ci/mM) was then added to each culture at the end of the incubation time, and cells were harvested after 4 h and washed on glass-fiber filters. Radioactivity trapped on the filters was measured in a liquid scintillation counter in counts per minute (cpm) [6].

Immunofluorescence Studies

Samples of EDTA-anticoagulated blood were drawn at 8 A.M. before administration of drugs. Sample were immediately stored on ice for a maximum of 2 h. Antibodies to the endotoxin receptor CD4+ (Mr59 kDA, Coulter Krefeld, Germany), CD8+ (32-kDA α-subunit, Coulter, Krefeld, Germany) or the respective isotypic controls were reacted under saturating conditions on ice with 100 μL of whole blood for 20 min. Leukocytes were fixed using the Coulter Q-Prep workstation. Cells were then washed once with cold phosphate buffer solution and immunofluorescence was analyzed in a flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis

Data are expressed as means $\pm$SD. Differences among groups were analyzed using the Students t-test and the Mann–Whitney U-test. Regression analysis was performed to evalute the independent relations between parameters. A two-tailed value $p < 0.05$ was considered as statistically significant.

Fluorescent Characteristics of Probe ABM

The maximum of the ABM fluorescence spectrum did not change in ly from patients with RA compared to those from healthy persons (the control group) (Fig. 1).

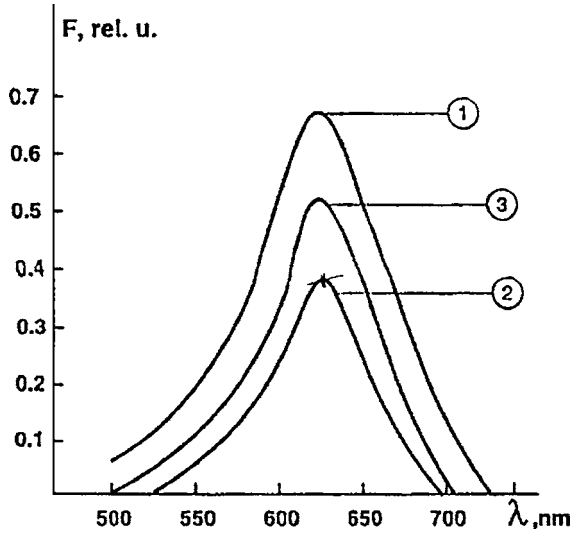

Fig. 1. Spectrum of the fluorescence emission of the ABM probe (λ_{ex} 470 nm) in suspension: (1) Patients with seronegative rheumatoid arthritis ($n = 49$); (2) Patients with seropositive rheumatoid arthritis ($n = 36$); (3) Healthy donors ($n = 41$). Note: Spectra represent mean values for the groups, concentration of ABM in the samples is 19.6 μM.

The binding of the ABM probe to the ly membranes, estimated by the ABM fluorescence intensity, was significantly increased in seronegative patients and decreased in seropositive patients as compared to the healthy donors (Table I).

Differences in the binding or partitioning of ABM in the membranes is confirmed by distinctively lower (seropositive RA patients) and higher (seronegative RA patients) amount of free probe left in the supernatant compared with the data obtained from practical healthy donors (Table I).

Experiments was performed to evolute the dependence of ABM fluorescence intensity on its concentration in RA patients ly suspensions (Fig. 2). Results testify, that seronegative RA patients Ly is more sensitive to the compound ABM.

These membrane alterations can be quantified by measuring the changes of membrane fluidity, using fluorescence anisotropy. As shown in Table II the seropositive RA patients cells tested posess distinctively higher anisotropy values, but seronegative RA patients lower, but seronegative RA patients-lower anisotropy values then those healthy donors. It is thus proposed that seronegative RA patients Ly membrane are in a more fluid state than seropositive RA one.

It is also confirmed by

$$y = 14.70 \times -2.24 \quad \text{(seroneg. RA patients)}$$
$$y = 4.85 \times -1.05 \quad \text{(seropos. RA patients)}$$
$$y = 6.53 \times -1.15 \quad \text{(healthy donors)},$$

where y is fluorescence intensity, x-anisotropy.

This finding indicate a different membrane alterations (i.g.-packing of lipid acid chaines) in the observed groups of patients.

It must be stressed that ABM fluorescence intensity correlates not only with the anisotropy ($r = 0.97$), but also with the concentration of membrane bound probe ($r = +0.94$) and amount of free probe in the supernatant ($r = 0.90$) (Table I).

Lymphocyte Number and Subpopulations

The number of Ly in blood of RA patients was significantly decreased as compared to healthy persons, particularly in patients with high RF titre (Table I). The $CD4^+/CD8^+$ ratio in peripheral blood was significantly increased in seronegative RA patients, but significantly decreased in seropositive RA patients. Among these groups of RA patients, a direct relationship between F and the ratio $CD4^+/CD8^+$ ($r = +0.80$) was observed.

Table I. Special Characteristics of ABM ($\lambda_{max} - 630$) Bound with the Membrane; Number and Subpopulations of *T*-Lymphocytes in RA Patients ($M \pm SD$)

Group under study	F 630 nm, au	Concentration of membrane-bound probe (μM)	Concentration of free probe in the supernatant (μM)	Number of Ly in 1 mL of blood plasma	$CD4^-/CD8^+$
Healthy donors	0.51 ± 0.02	3.30 ± 0.10	16.27 ± 0.28	$8.8 \times 10^5 \pm 6.5 \times 10^4$	1.93 ± 0.13
Patients with seronegative RA	0.64 ± 0.03	5.19 ± 0.14	14.39 ± 0.24	$4.1 \times 10^5 \pm 3.8 \times 10^4$	2.79 ± 0.21
p_1	<0.05	<0.05	<0.05	<0.05	<0.05
Patients with seropositive RA	0.37 ± 0.02	1.96 ± 0.08	17.48 ± 0.31	$3.5 \times 10^5 \pm 3.4 \times 10^4$	1.6 ± 0.10
p_1	<0.02	<0.05	<0.05	<0.02	<0.05
p_2	<0.01	<0.02	<0.02	<0.05	<0.05

Note. *F*-fluorescence intensity; concentration of the probe added is 19.6 μM. p_1: level of significance (between the RA patients and healthy donors). p_2: level of significance (between seronegative and seropositive RA patients).

Proliferation Activity of PBMC

The Ly proliferation activity was estimated by the amount of DNA synthesis (radioisotope method) when induced by mitogen. The proliferation activity of blood Ly in RA patients depended on the RF type. In patients with the seronegative RA form the functional activity was significantly increased (Table II). In contrast, in patients with the seropositive RA form, manifested by progressed development of the disease, decreased functional activity of the cells was observed. There is a direct correlation between ABM fluorescence intensity and Ly proliferation activity ($r = +0.984$).

DISCUSSION

Recent studies of structure and function relationships in biological mernbranes have shown that membrane lipids (unsaturation of fatty acids, phospholipids/cholesterol ratio, etc.) play an important role in the regulation of cellular function [8–11].

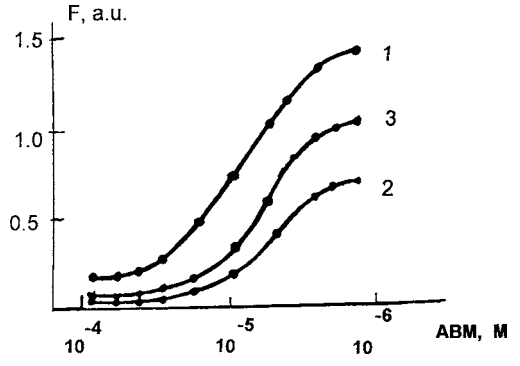

Fig. 2. Dependence of ABM fluorescence intensity on its concentration in mitogene-actived lymphocyte suspensions. Symbols as in Fig. 1.

Information exists on changes in the fatty acid composition of human ly during the period of blastic transformation and different pathologies [8]. Physical and chemical changes in membrane structure may contribute to the increased or decreased membrane anisotropy. Changes in membrane microviscosity of cells have been shown to correlate not only with physical and chemical changes of the mernbranes, but also with a decline of the cell responsiveness to mitogens (proliferation activity) [9,11]. Studies on lymphoid cells have demonstrated an enrichment of polyunsaturated a cids in the early steps of their activation, which has a significant positive influence on membrane anisotropy [8].

In cases of different pathologies the degree of fatty acid unsaturation decreases or increases, resulting in corresponding changes in membrane microviscosity [10].

In previous experiments a blue shift of ABM emission spectra was revealed both in liposomes enriched with cholesterol and in human peripheral blood Ly [5]. This in agreement with Shinitzky and Inbar's observation on the effect of cholesterol on liposome and cell fluidity [12]. In patients with RA, the ABM emission spectrum maximum was not changed in comparision with that in the healthy control group [5]. Therefore the absence of blue shift emission in the spectra indicates no significant cholesterol increase in Ly of patients with RA.

The above-mentioned literature data are in good agreement with the results of our previous studies using ABM of such pathologies as lung tuberculosis [5], gastrointestinal tract cancer [13], multiple sclerosis [14], chronic myeloleukemia, and B-cells lymphoid leukemia [15], as well as the check-up of the Chernobyl clean-up workers [16]. In cases of observed pathologies, the degree of fatty acid unsaturation increases or decreases, resulting in corresponding changes in membrane anisotropy. A correlation was found between ABM fluorescence intensity,

Table II. Spectral Characteristics of ABM and Anisotropy Index in Suspensions of Lymphocytes of Patients with RA

Group under study	Native cells		Activated cells (PHA: 10 μg/mL)		
	F, au	*r* anisotropy	F, au	*r* anisotropy	LBTR level (cpm)
Healthy donors	0.51 ± 0.02	0.254 ± 0.003	1.66 ± 0.10	0.157 ± 0.002	97984 ± 8747
Patients with seronegative RA	0.64 ± 0.03	0.196 ± 0.002	2.02 ± 0.12	0.124 ± 0.003	146052 ± 13461
p_1	<0.05	<0.02	<0.02	<0.05	<0.05
Patients with seropositive RA	0.37 ± 0.02	0.283 ± 0.004	0.52 ± 0.04	0.291 ± 0.004	39196 ± 3098
p_1	<0.02	<0.02	<0.02	<0.02	<0.05
p_2	<0.01	<0.01	<0.01	<0.01	<0.05

Note. Symbols as in Table I.

membrane anisotropy, and the proliferation activity of cells.

The seronegative and seropositive RA patents differed among themselves in spectral characteristics (Table I). According the above-mentioned, the obtained results can be explained by the qualitatively different changes in the structural and functional properties of cells, which is confirmed by changes in the anisotropy, and the partiotining of ABM in the membranes, proliferation activity.

The Ly count in blood of the RA patients was significantly decreased compared to that in healthy persons, and this effect was more pronounced in patients with a high RF titre (Table I). The $CD4^+/CD8^+$ ratio in peripheral blood was significantly increased in seronegative RA patients and significantly decreased in seropositive RA patients. A lower Ly count and disordered T-cells differentiation in RA has been described also by other authors [3,14]. We suggest that the general decrease in the lymphocyte count in blood can be due to some extent to a decrease of the T-lymphocyte level. Primary T-cell selection in RA patients has functional importance for the generation of synovium-specific T-cell responses. Disordered T-cells differentiation (ratio $CD4^+/CD8^+$) plays a key role in immunoregulation of the immune response and contributes to the typical inflammation in RA. CD4+ cells from RA manifest an intrinsic abnormality in their a bility to differentiate into specific cytokine-producing effector cells, which might contribute to the characteristic chronic (auto) immune inflammation in RA [17,18].

Summarizing the presented data its should be stressed that the ly functional activity revealed by the radioisotope method was different between patients with seronegative and seropositive RA forms. These changes can be explained as compensatory increase of the functional activity of the Ly in patients with seronegative RA. In patients with seropositive RA the decreased number of Ly in blood was coupled to their decreased activity.

The observed changes of the studied parameters are coupled to alterations of the cellular mechanisms of immunity regulation. The simultancous decrease of the functional activity of ly, decreased their total number in blood plasma and the decrease of the $CD4^+/CD8^+$ ratio in the seropositive RA patients, indicate greater alterations of immune regulation processes as compared to the seronegative RA: in the latter case the Ly deficiency is compensated to some extent by their increased proliferation activity.

The parameters of the physicochemical status of the Ly membranes and of the cell functional activity can be used as additional criterion for the evaluation of the degree of RA activity supplementary to the RF test.

The mentioned parameters correlate with clinical manifestations of the disease. The medical examination revealed that, in patients with the seropositive RA form the clinical manifestations were much more pronounced as compared to the seronegative group. Seropositive patients had intensive pain in articulations, lengthy morning constraint and rigidity coupled to pronounced proliferate phenomena. X-ray examination revealed significant deformation of articulations with pronounced destructive alterations.

The results show that the fluorescent probe ABM may be useful for screening alterations of the structural and functional properties of cells during pathologies.

This fluorescence-based method is less expensive and time consuming, nonhazardous, technically simple, sensitive and convenient than other methods (radioisotope, immunofluorescent, microscopic, flow cytometry, etc.)

The probe ABM is commercially available (please contact the authors).

REFERENCES

1. А. Маитулис (1985) *Современные методы диагностики ревматических заболеваний*, [Modern Methods in Diagnostics of Rheumatic Diseases]. Вильнюс, Моклас. (in Russian).

2. G. V. Bail and W. J. Koopmen (1986). *Clinical Rheumatology*, Saunders, Philadelphia, 367 pp.
3. A. Skapenko, J. Wendler, P. E. Lipsky, J. R. Kalden, and H. Schulzekoops (1999). Altered memory T-cell differentiation in patients with early rheumatoid arthritis. *J. Immunol.* **49**(4), 298–304.
4. J. R. Lakowicz (1984). *Principles of Fluorescence Spectroscopy*, Plenum, New York.
5. I. Kalnina and I. Meirovics (1999). A new fluorescent probe, ABM; properties and application in clinical diagnostics. *J. Fluoresc.* **9**. 27–32.
6. D. P. Stitas (1999). *Basic and Clinical Immunology,* 7th ed., Appleton and Lange, pp. 263–283.
7. D. B. Duncan (1970). Qucry multiple comparison methods for comparig regression coefficient. *Biometrics* **26**, 141–143.
8. A. Anel, K. Noval, B. Gonsalez, J. M. Torres, Z. Mishal, J. Uriel, and A. Pineiro (1990). Fatty acid metabolism in human lymphocytes. I. Time-course changes in fatty acid composition and membrane fluidity during blastic transformation of peripheral blood lymphocytes. *Biochim. Biophys. Acta* **1044**, 323–331.
9. L. A. Huber, G. B. Xu, G. Jurgens, G. Bock, E. Buhier, K. F. Gey, D. Schonitzer, K. N. Trail, and G. Wick (1991). Correlation of lymphocyte lipid composition, membrane microviscosity and mitogene response in the aged, *Eur. J. Immunol* **21**, 2761–2765.
10. M. Shinitzky (Eds.) (1984). *Physiology of Membrane Fluidity*, CRC Press, Boca Raton, FL, pp. 1–52.
11. В.А. Извеков (1991) Липиды мембран и функции иммунокомпетентных клеток в норме и патологии. (Membrane lipids and functions of immunocompetent cells in norm and pathology) Успехи совр. биол., 111(**4**) 577-590 (in Russian)
12. M. Shinitzky and M. Inbar (1974). Difference in microviscosity induced by different cholesterol levels in the surface membrane lipid layer of lymphocytes and malignant lymphoma cells. *J. Mol. Biol.* **85**, 603.
13. L. Klimkane, L. Kalnina, L. Engele, I. Jaunalksne, S. Donina, G. Zakenfelds, and I. Meirovics (1999). in A. Kotyk (Ed.), *Third Conference on Fluorescent Microscopy and Fluorescent Probes*, Espero Publishing, Prague, pp. 339–345.
14. I. Kalnina, M. Metra, L. Licitis, and I. Meirovics (1999). in A. Kotyk (Ed.), *Third Conference on Fluorescent Microscopy and Fluorescent Probes*, Espero Publishing, Prague, pp. 295–300.
15. R. Bruvere, N. Gabruseva, I. Kalnina, G. Feldmane, and I. Meirovics (2003). Fluorescent characteristics of blood leukocytes of patients with malignant and nonmalignant diseases. *J. Fluoresc.* **13**(2), 149–156.
16. I. Kalnina, N. Gabruseva, R. Bruvere, T. Zvagule, O. Heislere, A. Volrate, G. Feldmane, and I. Meirovics (2001). Phenotypical characteristics of leukocyte membranes in Chernobyl clean-up workers from Latvia: Use of the fluorescent probe ABM. *Proc. Latvian Acad. Sci., Serb.* **55**(1), 6–13.
17. T. Namekawa, U. G. V. Vamer, J. J. Goronzy, and C. M. Weyand (1998). Functional subsets of CD-4 T-cells in rheumatoid synovitis. *Arthr. Rheumat.* **41**(12), 2108–2116.
18. H. Y. Yang, H. Rittner, C. M. Weyand, and J. J. Goronzy, (1999). Aberration in the primarily T-cells receptor repertoire as a predisposition for synovitis inflammation in rheumatoid arthritis. *J. Invest. Med.* **47**(5), 236–245.