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QUARTERLY

Incorporation of stilbazolium merocyanines into human leukocytes measured by flow cytometry*

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Human peripheral blood leukocytes were incubated with thirteen various merocyanines of the stilbazolium betaine type and the fluorescence intensities of the cells were measured by flow cytometry. The fluorescence intensity of lymphocytes, monocytes and granulocytes depended on the time and temperature of incubation with the dyes. An increase in the incubation temperature enhanced the fluorescence intensity whereas washing of the cells after incubation had little influence on the observed emission. This points to incorporation of the dye molecules into the cell membrane. From the measured fluorescence intensities corrected for relative fluorescence yields, the relative efficiencies of incorporation into the cells of the various merocyanines tested were evaluated. The efficiency was dependent on the type of the cells and the length and side groups of the merocyanine molecules studied.

Merocyanines of the stilbazolium betaine type have attracted much attention because of their toxic effects on tumor cells *in vitro* [1, 2]. This cytotoxic action could be facilitated by the ability of merocyanine to penetrate into the phospholipid bilayer of the cellular membrane [3]. Recently [4] we have found that the membranes of the human peripheral blood mononuclear cells incorporate stilbazolium merocyanine dyes *in vitro*. After incubation with the dye the cells exhibited a fluorescence characteristic of merocyanines emission [4]. Various merocyanines penetrate the membrane with different efficiency [3]. In the present work, to verify whether it is possible to study the process of the dye incorporation using flow cytometry, we investigated by this method the fluorescence of different types of leukocytes incubated with several types of merocyanines.

MATERIAL AND METHODS

Dyes. The stilbazolium merocyanine dyes used in these investigations, denoted by capital letters, are presented in Table 1. In the text the derivatives are referred to as mero- with an appropriate letter (cf. Table 1). Melting points, IR and NMR spectra and elemental analysis of these dyes were described earlier [5, 6]. The dyes were dissolved in methanol (0.01 M) and stored at –12°C before use.

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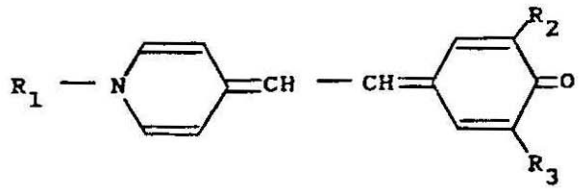
Abbreviations used: F, fluorescence intensity; FBS, foetal bovine serum; PHA, phytohemagglutinin.

Table 1
Chemical formula of stilbazolium merocyanines



Notation	R_1	R_2	R_3
A	$-(CH_2)_6-OH$	-H	-H
B	$-(CH_2)_{11}-OH$	-H	-H
C	$-(CH_2)_6-OH$	$-CH_3$	$-CH_3$
D	$-(CH_2)_6-OH$	$-C(CH_3)_3$	$-C(CH_3)_3$
O**	$-(CH_2)_6-OH$	$-C(CH_3)_3$	$-C(CH_3)_3$
E	$-(CH_2)_{11}-OH$	$-C(CH_3)_3$	$-C(CH_3)_3$
F	$-(CH_2)_{15}-CH_3$	$-C(CH_3)_3$	$-C(CH_3)_3$
G	$-(CH_2)_6-OH$	$-OCH_3$	$-OCH_3$
J*	$-(CH_2)_{10}-COOH$	-H	-H
K*	$-(CH_2)_{12}-OH$	-H	-H
L*	$-(CH_2)_{15}-CH_3$	-H	$-OCH_3$
L_1*	$-(CH_2)_{11}-OH$	$-OCH_3$	-OCH3
N*	$-(CH_2)_{10}-COOH$	$-C(CH_3)_3$	$-C(CH_3)_3$

*, HCl salt; **, $HClO_4$ salt

Cells. Usually, whole heparinized blood samples remaining after routine analysis were incubated with merocyanines. In some experiments, cells derived from lymphocyte cultures were used.

For this purpose, mononuclear cells were isolated from heparinized peripheral blood of an adult healthy donor by sedimentation through a Gradisol (Polfa, Poland) gradient (d = 1.077 g/cm), according to Bøyum [7]. Cells (2×10^5 per well) were cultured on 96-well flat-bottom microplates (Sterilin, Great Britain) in a total volume of 0.2 ml of Eagle's medium supplemented with 10% foetal bovine serum (FBS) and gentamycin (20 μg/ml).

Phytohemagglutinin (PHA, HA 17, Wellcome, England, 1 μg/ml) was used as a mitogen. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Then the cells were collected, washed once with 0.9% NaCl supplemented with 10% FBS. The lymphoblasts were distinguished on the cytogram as a population of large cells expressing receptor for interleukine 2 (CD25) when stained for 45 min at room temperature with a monoclonal antibody against CD25 (Ortho-mune OKT 26 A). The contamination of this population by monocytes (as checked by Ortho-mune antibody against CD14) was lower than 3%.

Incubation with the dye. A merocyanine, 5 μl, dissolved in 0.01 M methanol was added to 5 ml of blood or 5 ml of cells suspended in 0.9% NaCl. The same volume of methanol was added to control samples. The incubation was carried out usually at 25°C for 1 h. In some experiments the temperature of incubation was changed (from 4° up to 37°C) and the time of incubation ranged from 0.5 h to 3 h. The results of fluorescence measurements for washed and unwashed samples were usually similar. Only the fluorescence of lymphocytes incubated with mero-A decreased slightly as a result of the washing. After the incubation samples were treated with 1 ml of the Ortho-mune lysing reagent.

Fluorescence measurements. The 3-fluorescence color flow cytometer (Cytoron Absolute, Ortho) equipped with an argon ion laser was used (Fig. 1). The fluorescence, excited at 488 nm, was measured through the orange band pass filter (563–607 nm) in the direction perpendicular to that of the exciting light and the cell's stream (Fig. 1). The intensity of emission of a given population of the cells was obtained from the Immunocount program (Ortho) using the

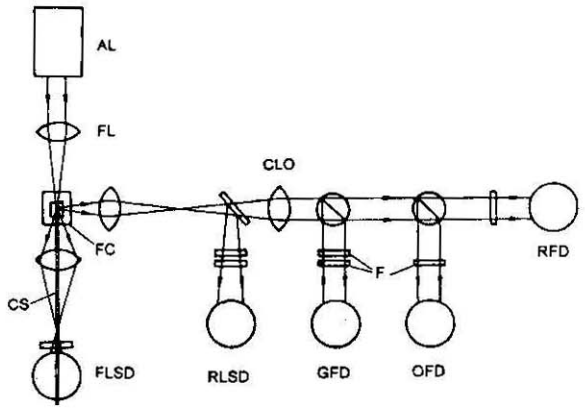


Fig. 1. Scheme of the cytometer.
AL, argon laser; FL, focusing lens; FC, flow cell; CS, cells stream; FLSD and RLSD, detectors of light scattered in the forward direction and at a right angle, respectively: GFD, OFD and RFD, detectors of fluorescence light (G, green; O, orange; R, red). F, filter(s); CLO, collimating objective.

gate analysis method [8]. The distribution of the population of fluorescent cells between various channels was thus determined. Examples of the distributions obtained for mero-A and mero-B are shown in Fig. 2. As a measure of the fluorescence intensity of a given population of the cells, the fluorescence due to the cells gathered in the "mean channel" was established. The number of cells counted in the "mean channel" was close to the mean value of the numbers of cells counted in all channels. The fluorescence intensities (Table 2) were proportional to light emission over whole spectral region measured. Therefore changes in the fluorescence spectra, which could be due to differences in positioning of the dye molecules in the membrane or on the cell surface could not be observed. In this situation the results concerning the same type of the cells could be treated only quantitatively. To compare, on the basis of fluorescence intensities, the efficiencies of incorporation of various merocyanines into the same type of the cells the relative yields of the fluorescence of the dyes (η/η_A) have to be known.

The fluorescence intensity (F) depends on the energy yield of emission (η): $F = cI_oA\,\eta$, where I_o is the intensity of the exciting light; A, absorption of the light in a sample; c, a coefficient dependent on the geometry of the system applied.

The whole set of measurements for various dyes in methanol was taken at the same A, I_o and c values. The excitation from the front of the samples enabled to neglect the reabsorption and secondary fluorescence. In this situation the ratio of fluorescence intensities was equal to the relative yields of fluorescence in methanol. The absolute yields of fluorescence of merocyanines in methanol and in the cells are different, but from a comparison of relative yields in solution and in polymer films it followed that the relative yields were only slightly altered on changing the medium. Therefore the relative yields obtained for methanol solution were used for evaluation of the incorporation efficiency (Table 2). The positions of absorption and fluorescence bands of the investigated merocyanines were similar, therefore the same excitation wavelength (488 nm) and pass filter could be used. The emission data corrected for the fluorescence yields, perimt to evaluate the relative efficiencies of dye incorporation in a given type of the cells (Table 2).

The population of the cells present in a sample was established also with the use of a flow cytometer by measuring of both the forward- and the right-angles-cattered light (not shown).

RESULTS AND DISCUSSION

From the results presented in Table 2 it follows that mero-B and mero-K which have the same side groups H at positions R_2 and R_3 exhibited a higher yield of incorporation than a similar molecule, mero-A with a shorter chain at position R_1. The molecules with the bulky t-butyl side groups at R_2 and R_3 (mero-D, mero-E, mero-F, mero-N*, mero-O**) were not efficiently incorporated into the cells. As it has been found previously [1] such bulky groups, efficiently prevent the formation of ground state aggregates which can diminish the efficiency of the dye incorporation. t-Butyl groups perturbed the incorporation even in the case of a

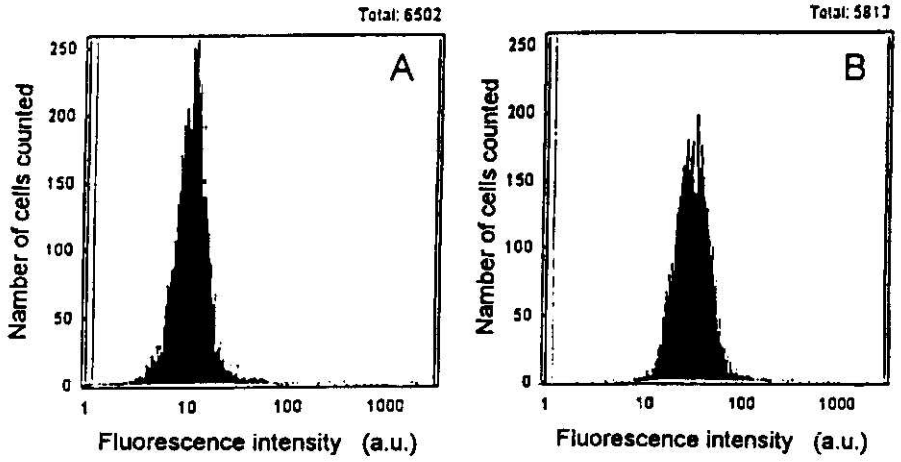


Fig. 2. The cytograms of fluorescence intensity gated around granulocytes incubated during 1 h (25°C) with mero-A and mero-B. Cells stained with mero-A and mero-B show a different level of fluorescence intensity.

Table 2

Evaluation of relative numbers of merocyanine dye molecules (N) incorporated into various types of leukocytes.

F, fluorescence of cells obtained from "mean channel" of the cytometer; η/η_A, relative yield of dye fluorescence. Incubation: 1 h at 25°C.

Type of merocyanine (according to Table 1)	η/η_A	Lymphocytes		Monocytes		Granulocytes	
	± 0.05	F ± 1	N ± 0.05	F ± 1	N ± 0.05	F ± 1	N ± 0.05
A	1	17	1	49	1	40	1
B	0.58	60	5.88	157	5.52	88	3.79
C	18.84	7	0.02	18	0.02	21	0.03
D	11.97	5	0.02	12	0.02	16	0.03
O**	7.28	4	0.03	16	0.14	16	0.05
E	7.88	4	0.03	9	0.02	16	0.05
F	12.56	4	0.02	11	0.02	13	0.03
G	1.04	5	0.29	8	0.16	11	0.26
J*	11.51	20	0.16	53	0.09	38	0.08
K*	0.51	27	3.18	66	2.64	51	2.50
L*	3.93	48	0.71	147	0.76	133	0.85
L_1*	1.11	19	1.12	35	0.64	37	0.83
N*	6.78	2	0.02	11	0.03	11	0.04

monomeric dye (Table 2). The -OCH_3 groups located at position R_3, or both at R_2 and R_3, lowered the incorporation yield to a smaller degree than did t-butyl groups.

The kinetics of the dye incorporation obtained from the fluorescence data after various incubation times (not shown) were faster for the dye with a shorter chain at R_1, but the dyes with longer chains exhibited a higher final dye concentration.

Table 3

Incorporation of merocyanines dyes into lymphocytes cultured in vitro and incubated with these dyes (10^{-4} M).

Notation as in Table 2. Incubation 1 h at 25°C.

Mero-cyanine	Lymphoblasts		Resting lymphocytes	
	F	N	F	N
A	33	1	15	1
B	40	2.09	00	0.00
C	30	0.05	13	0.05
J*	33	0.09	14	0.08

The fluorescence intensities and efficiencies of incorporation for lymphocytes cultured *in vitro* are shown in Table 3. The difference between the dye incorporation into resting cells and into lymphoblasts was observed only for some of the dyes (in Table 3 for mero-B).

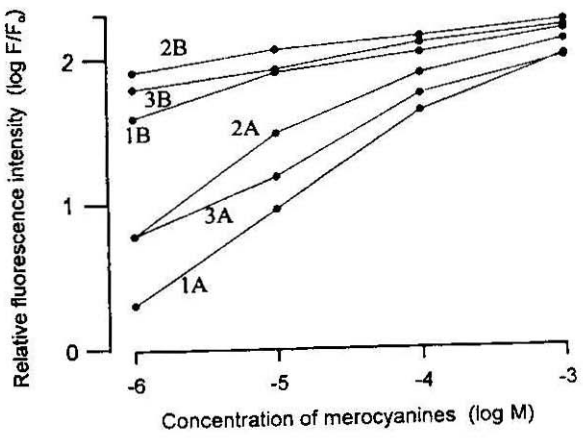


Fig. 3. The dependence of the "mean channel" fluorescence intensity on the concentration of the dye used.

The cells were incubated for 1 h at 25°C. A, Mero-A; B, mero-B; 1, lymphocytes; 2, monocytes; 3, granulocytes.

Figure 3 shows the dependence of fluorescence intensity of leukocytes on the concentration of mero-A and mero-B dyes used for incubation of various types of cells. For all populations of the cells the fluorescence intensity of the cells incubated with mero-B was higher than that of the cells incubated with mero-A. In the latter, the fluorescence was dependent more strongly on the type of the cells and on the dye concentration than was the emission of the cells incubated with mero-B.

The dependence on the time of incubation (not shown) in the presence of these two dyes was also different: the plateau of fluorescence intensity with mero-B was reached within 0.5 h whereas with mero-A, after 3 h, but in the latter case the selectivity of its incorporation into various types of the cells was higher than with mero-B.

In all three types of the cells the fluorescence intensity increased with an increase in temperature (Fig. 4). This strongly suggests that the dye molecules were incorporated into the cell membrane.

The higher emission intensity observed for the larger cells (Fig. 4) could be, at least partially, due to the cell dimensions as, on the larger surface of a fluorescent membrane the emission intensity could be increased.

Within the same type of the cells (e.g., monocytes) the temperature dependence of the mero-A and mero-B emission was different. This shows that the same changes in the membrane structure due to the increase in temperature, influence in a varying degree the incorporation efficiency of different dyes.

The presented results show that flow cytometry can be used in investigations on incorporation of various merocyanines into different types of the cells. An additional advantage of this method is the opportunity to evaluate the distribution of dimensions of the measured cells which is all the more important as this distribution is altered if the cells become fragmented. It is also possible to check whether only one type of the cells is present in a sample.

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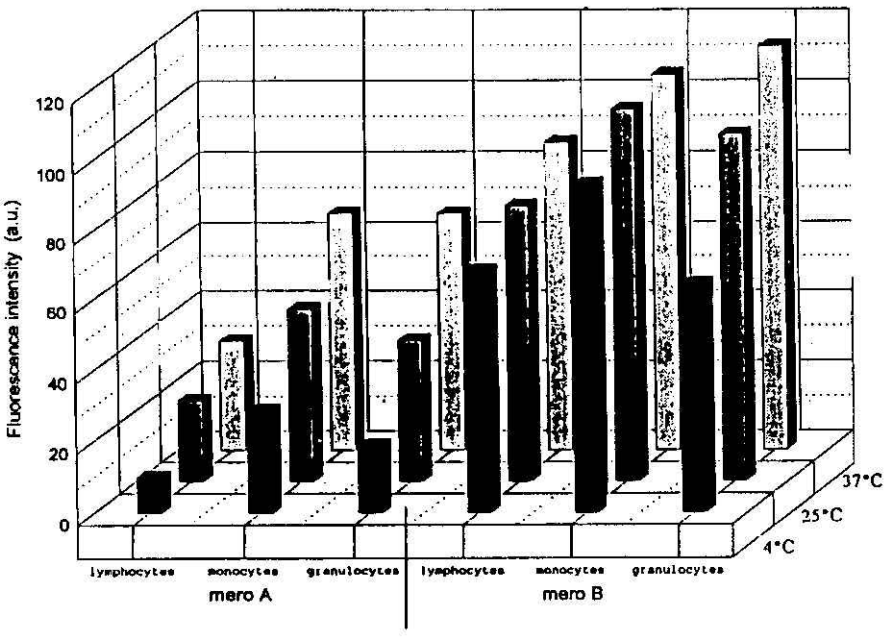

Fig. 4. The dependence of fluorescence intensity on temperature of incubation with mero-A and mero-B (1 h) for various types of leukocytes.

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