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RYODIPINE – A NEW FLUORESCENT PROBE FOR DETECTING DIFFERENCES BETWEEN LYMPHOCYTES

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Several years ago a fluorescent method was suggested for distinguishing between lymphocyte populations [7, 9, 14]. This method was based on the use of the fluorescent probe MBA (3-methoxybenzanthrone), on staining with which B lymphocytes fluoresce more brightly than T lymphocytes. Later, diagnostic tests have been created on the same principle, by which it is possible to detect bronchial asthma [5, 8] and some malignant blood diseases [1, 6]. In these cases the diagnosis is based on recording the intensity of fluorescence (F) of single leukocytes in human blood, stained by the MBA probe, and comparing histograms of distribution of the cells with respect to F in normal subjects and in the pathological state.

The further development of the technique of fluorescent probing of cells necessitates the search for new fluorescent probes. The investigation described below is one step in this direction. It demonstrates that the fluorescent hypotensive agent ryodipine (phoridone) is superior to the familiar fluorescent probe MBA in a number of parameters relating to staining of lymphocytes. These data encourage the hope that ryodipine will succeed in replacing the MBA probe as a means of identifying T and B lymphocytes in the diagnosis both of bronchial asthma and of malignant blood diseases. Such a change would make these methods more reliable.

EXPERIMENTAL METHOD

Lymphocytes were isolated from the thymus and Peyer's patches of noninbred albino rats by the standard method [10]. Cell suspensions were prepared in Hanks' solution, pH 7.4 (Institute of Poliomyelitis and Virus Encephalitis, Moscow) and the cell concentration was determined in a Goryaev's counting chamber. To stain the cells with the fluorescent probe MBA (synthesized in B. M. Krasovitskii's Laboratory, "Monokristall" Research and Production Combine, Khar'kov) and with ryodipine (synthesized by V. V. Kastron at the Institute of Organic

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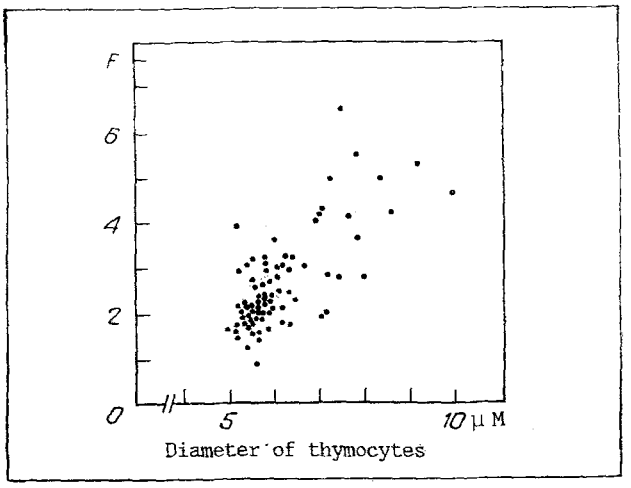

Fig. 1. Correlation between intensity of F of rat thymocytes stained with ryodipine and their size. Concentration of ryodipine 20 μM, of cells 4 × 10^7/ml.

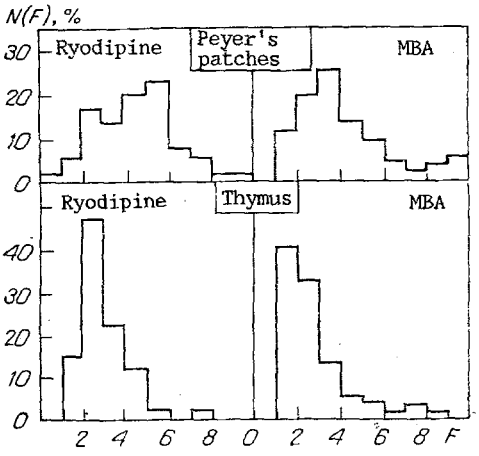

Fig. 2

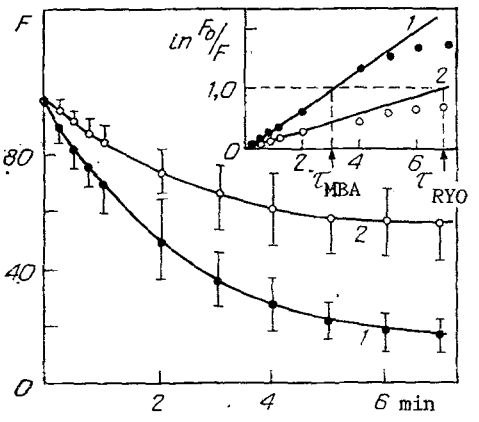

Fig. 3

Fig. 2. Histograms of distribution of rat lymphocytes by intensity of F of cell-bound ryodipine (a, b) and MBA probe (c, d). Lymphocytes isolated from Peyer's patches (a, c) and thymus (b, d). F) fraction of cells (in %) having the given value of F; concentration of cells 5 × 10^6/ml, of ryodipine and MBA 20 μM. In each case, F was measured in 100 cells.

Fig. 3. Changes in intensity of F of thymocytes stained with MBA probe (1) and ryodipine (2) after exposure to exciting beam of light. Concentration of cells 2.5 × 10^7/ml, of MBA and ryodipine 20 μM. Each point represents mean value of F for 10 cells ± standard deviation (calculated as $\sqrt{\sigma^2}$); F_0) intensity of fluorescence at beginning of measurement.

Synthesis, Academy of Sciences of the Latvian SSR), the corresponding volume of the initial solution (2 mM) of MBA (in dimethylformamide) and of ryodipine (in ethanol) was added to 1 ml of cell suspension, mixed on a magnetic mixer, by means of a microsyringe.

Photometry of the cells was carried out on a "Lyumam I-2" cytofluorometer (Leningrad Optico-Mechanical Combine). The source of exciting light was a DRSh-250-3 mercury lamp. F of ryodipine was excited in the 365 nm region, and of MBA in the 436 nm region. When F was recorded, the interference filters of an FMÉL-1 attachment with maxima of transmittance of 446 nm for ryodipine and 520 nm for MBA, were used. For photometry, a diaphragm selecting an area of the specimen 12.5 μ in diameter, was brought up to the cell. The scale on which the intensity of F was expressed was the same in all measurements. Histograms of distribu-

tion of the cells by their F value were constructed as in [12]. In the experiments to determine correlation between the intensity of F of cells stained with ryodipine and their size, after F of the cells had been recorded the diameters were measured in transmitted light by an ocular micrometer. To estimate the significance of the coefficient of paired correlation (r) tables given in [13] were used.

In experiments to study photodestruction of the dyes in the cells, F of two cells at opposite corners of a coverslip along the diagonal were recorded on the same slide.

EXPERIMENTAL RESULTS

A previous study of the intracellular localization of the fluorescent hypotensive agent ryodipine showed that a high proportion (at least 80%) of its F in thymocytes is due to membrane-bound ryodipine [3]. This suggested that by measuring F of lymphocytes stained with ryodipine, the quantity of cell membranes could be approximately (but quickly) estimated.

That this suggestion is correct is shown by the results given in Figs. 1 and 2. Figure 1 demonstrates significant correlation ($r = 0.74$; $p < 0.001$) between the size of the thymus cells and their F after staining with ryodipine. This correlation can be explained by the different morphology of the thymus cells. We know that 95% of thymus cells are T lymphocytes [4], among which may be distinguished large (8-10%), medium (35-40%), and small (50-60%) forms [11], differing from one another not only in size, but also in the number of intracellular membranes. Intracellular membranes are most highly developed in large T lymphocytes, less so in small (these are cells with a high nucleo-cytoplasmic ratio) [11].

Different subpopulations of lymphocytes also are known to differ in the number of their cell membranes: in B cells the surface area of the membrane is 1.2 times greater than in T cells [2]. Differences between lymphocytes belonging to different populations can also be seen with respect to F of ryodipine. Histograms of distribution of thymocytes and cells of Peyer's patches (about 70% of them are B lymphocytes [7]) by intensity of their F after staining with ryodipine are given in Fig. 2. Clearly the lymphocytes of the patches give brighter fluorescence: the mean intensity of F in cells of Peyer's patches was 1.54 times greater than in thymus cells. It is interesting to note that similar histograms of thymocytes and cells of Peyer's patches were obtained on staining with the probe MBA (Fig. 2). Under these circumstances cells of Peyer's patches also fluoresce more brightly (1.6 times) than thymus cells. This means that much the same information about lymphocytes can be obtained with the aid of ryodipine as of MBA.

It was stated above that the MBA probe can be widely used to differentiate lymphocyte populations (to distinguish B from T cells [9, 7, 14]) and, most important of all, in the diagnosis of various diseases [1, 5, 6]. However, experience of work with MBA shows that this probe has a number of serious disadvantages, which interfere greatly with its use. MBA is rather toxic for cells and its F in lymphocytes depends on the cell concentration in the working suspension [14]. Accordingly, a definite ratio of MBA to cell is recommended for di-

TABLE 1. Comparative Characteristics of Staining of Lymphocytes with MBA probe and Ryodipine

Characteristics compared	Probe	
	MBA	ryodipine
Mean intensity of F ± error of mean (in mV of recording system) under identical conditions (signal amplification, concentrations of cells and probes). F measured in 100 cells from:		
thymus	53,2±3,0	58,5±2,2
Peyer's patches	84,5±4,4	90,2±3,7
Standard deviation of normalized (equalized with respect to mean values) F:		
σ_F – for thymocytes	33,0	21,8
σ_F – for cells from patches	46,5	36,7
Ratio of mean intensities of F in lymphocytes from Peyer's patches and thymus: $F_{patches}/F_{thymus}$	1,59±0,12	1,54±0,09
Interval within which F of cells is independent of ratio between concentrations of probe/cell (in moles/cell)	$(1—1,25)\cdot10^{-15}$	$(1—10)\cdot10^{-15}$
Change in intensity of thymocytes during exposure to exciting beam of light:		
Degree of photodegradation of probe in thymocyte after 7 min (%)	82±2	40±6
Time constant of photodegradation (in min)	3	7

agnostic purposes, which has been found to be optimal within a very narrow range: (1.00-1.25) × 10^{-15} moles MBA per cell [1]. Our experience of working with this probe also shows that the low photostability of MBA is the factor causing the greatest difficulty during its use: cells stained with it and exposed to an exciting beam of light quickly "burn out" (Fig. 3).

Experiments with the use of ryodipine as the stain for lymphocytes showed that it is largely free from the disadvantages inherent in MBA. In the case of ryodipine the range of the dye/cell ratio, within which F of the lymphocytes is independent of cell concentrations, is an order of magnitude greater than for MBA: (1-10) × 10^{-15} moles/cell. In the concentrations used in this work, ryodipine did not affect the viability of thymocytes for 1 h (according to the trypan blue test). Moreover, previous experiments with simultaneous staining of lymphocytes with ryodipine and the DSM probe [3] showed that F of DSM, reflecting the energy state of the cells, is not reduced in the presence of ryodipine.

Another important advantage of ryodipine over MBA is that it is less likely to "burn out." Whereas F of MBA in thymocytes exposed to an exciting beam of light falls by 82 ± 2% in the course of 7 min, F of cells stained with ryodipine falls only half as much: by 40 ± 6% (Fig. 3). The time constant of the "burning out" process (τ) for ryodipine is correspondingly 2.3 times greater than for MBA (τ is 7 min for ryodipine and 3 min for MBA; Fig. 3). At the same time, it will be clear from the inset to Fig. 3 that during 3 min MBA actually "burns out" e times, whereas ryodipine "does not burn out" e times in the course of 7 min. "Nondegrading" ryodipine is perhaps present in the cells:

$$F/F_0 = (1-\beta) + \beta_{exp}(-t/\tau),$$

where β is the fraction of degrading and $(1-\beta)$ the fraction of "nondegrading" ryodipine. In this case, all that can be said is that the initial velocity of the "burning out" process $(dF/dt)\cdot F_0$ for ryodipine is $^7/_3$ times lower. Under these circumstances, only 0.18 × F_0 in the case of MBA and 0.6 × F_0 in the case of ryodipine remain after 7 min, a difference of $^{10}/_3$ times. It can be concluded from these observations that ryodipine is less subject to photodegradation than MBA.

On the basis of these results ryodipine can be recommended as a new fluorescent probe. Its F value in lymphocytes reflects basically the number of cell membranes. Compared with the familiar fluorescent probe MBA, which conveys similar information about lymphocytes, ryodipine has a number of advantages (Table 1). First, F of ryodipine in cells is much less dependent on their concentration in the working suspension. This means that approximate values of the cell concentration in the working suspension will suffice. Second, ryodipine is less subject to photodegradation than the MBA probe, so that F of a large number of cells can be measured on the same slide, if ryodipine is used. It is therefore possible that ryodipine will be used in the future for those diagnostic problems for which the MBA probe is nowadays used, and also in other cases when the number of membranes in white blood cells is an important parameter.

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