METHODS

A FLUORESCENT PROBE TO INDICATE DIFFERENCES BETWEEN BLOOD T AND B LYMPHOCYTES

G. E. Dobretsov, L. G. Korkina,
G. Walzel, Yu. I. Zimin, É. M. Kogan,
and Yu. A. Vladimirov

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A new physicochemical marker for human peripheral blood lymphocytes is suggested. The lymphocytes were stained intravitally with the fluorescent probe 3-methoxybenzathrone and examined microfluorometrically. The blood lymphocyte population was shown to be heterogeneous. Two main groups of cells, differing in their intensity of fluorescence, are distinguished. Immunologic fractionation of the lymphocytes showed that one group of cells consists of T lymphocytes and the other of B lymphocytes.

KEY WORDS: T and B lymphocytes; identification; fluorescent probe.

Differences in the surface characteristics of human blood T and B lymphocytes [12] lie at the basis of methods used to identify and count immunocompetent cells. However, in some immunodeficient states and in diseases accompanied by manifestations of atypical proliferation of lymphocytes, the stability of cell markers is disturbed and difficulties arise in the use of heteroantisera. These circumstance explain the need for a search for new markers for human T and B lymphocytes. In recent years fluorescent probes have become widely used as a method of studying the structure of cell membranes and subcellular particles [1, 2, 4, 6, 8]. Fluorescent probes are very sensitive to differences in membrane structure. It might therefore be suggested that these probes would help to reveal structural differences between T and B cells in human peripheral blood. Of the various fluorescent probes, 3-methoxybenzanthrone was chosen, for, as shown previously [1, 2], it is highly sensitive to conformational changes in proteins and membranes.

EXPERIMENTAL METHOD

Isolation of Lymphocytes from Blood. Uncoagulated blood from donors aged 25-35 years was diluted 1:3 with medium No. 199. A 2.7% solution of EDTA, pH 7.2, was used as anticoagulant. Lymphocytes were isolated from whole blood in a single-step Ficoll-Urotrast gradient as described in [7] and the lymphocytes thus obtained were washed 3 times in medium No. 199.

The method described in [11] was used for fractionation of T and B lymphocytes. A suspension of mononuclear cells in a volume of 2-3 ml was mixed with an equal volume of a 0.5% suspension of sheep's erythrocytes (E) or of bovine erythrocytes treated with antiserum and complement (EAC). After the procedures required for optimal E and EAC rosette formation [3, 5, 9] the mixtures were layered separately above 2 ml of Ficoll-Urotrast solution and centrifuged for 30 min at 400g. Lymphocytes forming rosettes settled under these circumstances to the bottom of the tube. T lymphocytes remained in the interphase after sedimentation of EAC-rosettes and B lymphocytes remained after sedimentation of E-rosettes (E-RFC). In order to obtain an enriched suspension of T lymphocytes the residue of E-RFC was incubated at 37°C for 1 h. After dissociation of lymphocyte—E complexes, the cell suspension was fractionate in a Ficoll-Urotrast system. T cells were harvested from the interphase and the erythrocytes settled to the bottom of the tube.

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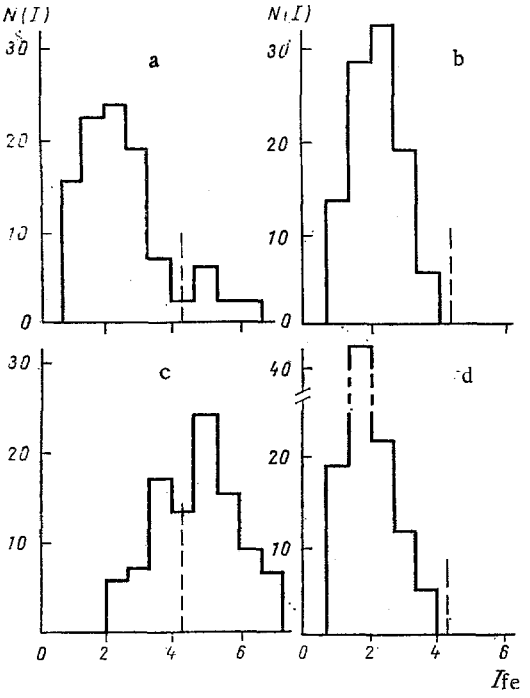

Fig. 1. Histograms of distribution of human blood lymphocytes by intensity of fluorescence of MBA probe bound with cells. a) Original lymphocyte suspension; b) cells remaining after removal of EAC-rosettes; c) cells remaining after removal of E-rosettes; d) cells obtained from E-rosettes by thermal dissociation of rosettes. N(I) denotes proportion of cells with given intensity of fluorescence (I_{fl}), in % of total number of cells.

Measurement of Fluorescence. For fluorochroming, 1 µl of a solution of 5 mM 3-methoxybenzanthrone (MBA) in dimethylformamide was added to 0.2 ml of a suspension containing 4×10^6 lymphocytes. The flux of fluorescence of MBA from one lymphocyte was measured. Fluorescence was excited at 405 nm and recorded at 540 nm on the ML-4 microfluorometer (LOMO) immediately after staining.

EXPERIMENTAL RESULTS

Blood lymphocytes stained with MBA give bright green fluorescence of their membranes. The cells differed in their intensity of fluorescence. Histograms of distribution of the cells by intensity of fluorescence are shown in Fig. 1a. Two maxima differing in intensity by 2-2.5 times can be seen on the histograms. Conventional differentiation of the cells into a "bright" group and a "less bright" group (shown by the broken line in Fig. 1a) reveals individual differences in the proportions of the two groups of cells (Table 1). The mean number of "bright" cells was 16%.

Investigation of the human peripheral blood lymphocyte population by different methods and, in particular, by determination of the electrophoretic mobility of single cells [10] or by the rosette-formation test [5], has shown that the proportion of B lymphocytes depends individually and on the donor's age and varies from 4 to 20% [10] or from 12 to 23% [5].

Comparison of the results of the present experiments with data on rosette formation suggested that the heterogeneity observed by the fluorescence method is due to the presence of T and B lymphocytes. To test this hypothesis, additional fractionation of human blood lymphocytes into T and B cells was carried out. If the EAC-forming cells (B lymphocytes) were removed, the histogram of the remaining cells was shown to have only one maximum (Fig. 1b). Its position corresponds to the maximum of the "less bright" cells on the histogram of the original lymphocyte suspension (Fig. 1a). A pure population of T lymphocytes obtained by temperature dissociation of E-rosettes also had only one maximum, corresponding to that of the "less bright" cells in the previous cases (Fig. 1d). Conversely, after removal of the E-rosettes, the remaining cells were grouped mainly around the maximum coinciding with the

TABLE 1. Content of "Bright" Cells in Human Blood*

Donor	Total number of cells tested	Number of "bright" cells	
		absolute	%
1	200	38	19
2	200	26	13
3	200	52	26
4	100	14	14
5	200	25	12.5
6	200	36	18
7	100	10	10

*Average content of "bright" cells is 16.25%

maximum of the "bright" cells on the combined histogram (Fig. 1c). However, among these cells there was an appreciable number of weakly fluorescent cells. These were evidently the so-called null-lymphocytes not forming E-rosettes yet, at the same time, not carrying markers of B cells.

The fluorescent probe MBA can thus reveal individual differences between peripheral blood lymphocytes. Depending on the intensity of fluorescence of MBA, all lymphocytes can be divided into two groups: bright and less bright. The T lymphocytes form the less bright group of fluorescent cells. The intensity of fluorescence of the B lymphocytes is on average 2-2.5 times greater than that of the T lymphocytes.

The results show that the fluorescent probe MBA can serve as an indicator of differences in the membranes of T and B lymphocytes. This suggests that fluorescent probes may prove useful for the diagnosis of pathology of the immune system.

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