Comparative Analysis of Accumulation of Chlorine e6 and Hematoporphyrin Derivatives in Subpopulations of Peripheral Blood Lymphocytes

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We studied accumulation of porphyrin photosensitizers chlorine e6, hematoporphyrin, and their derivatives by different lymphocyte subpopulations. The intensity of staining of B lymphocytes and natural killer cells with photosensitizers was higher compared to T lymphocytes. T cell subpopulation differed by their ability to bind photosensitizers. Relative accumulation of dimethyl esters of chlorine e6 and hematoporphyrin in cells surpassed that of nonesterified porphyrins.

Key Words: *photodynamic therapy; photosensitizers; lymphocytes; accumulation; flow cytometry*

Photodynamic therapy (PDT) of tumors is a modern organ-sparing method for the treatment of cancer patients. This method is based on selective effects of light radiation on tumor tissues and cells after administration of photosensitizers in nontoxic doses [7]. PDT is most effective for the treatment of solid tumors. This method can also be used for the therapy of patients with other diseases, *i. e.* immune disorders [10].

Unfortunately, little attention was given to the immunomodulatory effect of photosensitizers. PDT was used to prevent the graft-versus-host reaction (selective treatment of activated T cells) [8]. PDT of tumors can be followed by activation of antitumor immunity. It was confirmed by infiltration of tissues with lymphocytes and plasma cells and induction of immunoregulatory cytokines after administration of photosensitizers. PDT in low doses modifies antitumor activity of macrophages [12]. Previous studies showed that porphyrin pigments formed during photoirradiation decelerate the development of autoimmune diseases [10,14]. The effects of PDT are realized via changes in the expression of antigens and selective damage to several cell subpopulations. However, fine mechanisms of the immunomodulatory effect of photosensitizers remain unknown.

The efficiency of photodynamic treatment depends on intracellular photosensitizer concentration. The study of pigment accumulation in lymphocytes of various populations is an urgent problem. Photosensitizers capable of selective binding to several types of immunocompetent cells hold much promise for photomodulation of immune processes. These substances modulate activity of immunocompetent cells during the therapy of diseases associated with immune activation.

Here we studied accumulation of porphyrin pigments in peripheral blood lymphocytes of various subpopulations. Experiments were performed with photosensitizers hematoporphyrin (HP), chlorine e6 (Cl e6), and their dimethyl esters. Previous studies showed that these pigments are potent photosensitizers for PDT [2,4].

MATERIALS AND METHODS

We used Cl e6, Cl e6 dimethyl ester (N. D. Kochubeeva, Institute of Molecular and Atomic Physics,

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Belarussian Academy of Sciences), HP, and HP dimethyl ester (Porphyrin Products). Stock solutions of pigments were prepared in acetone or ethanol (10^{-3} M) and stored at 4°C.

Peripheral blood samples were taken from healthy donors and placed in silicon tubes containing anticoagulant (20 U/ml heparin). Mononuclear cells (MNC) were isolated by centrifugation in a Histopaque-1077 gradient (Sigma). Normal leukocytes were stained using monoclonal antibodies (MCA, Becton Dickinson) labeled with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) [13]. MCA (20 μl) were added to the sample (100,000-200,000 cells). Incubation was performed in dark at room temperature for 25-30 min. After incubation with antibodies, the cells were washed twice with phosphate buffered saline (300*g* for 5 min). Before cytofluorometry, the cell suspension was placed in RPMI-1640 medium containing fetal bovine serum (Sigma) and Cl e6 derivatives.

Accumulation of pigments in lymphocytes was studied on a FACS Vantage cytofluorometer (Becton Dickinson). The argon laser served as a source of excitation (λ=488 nm, 150 mW). Fluorescence of pigments was recorded with a LP560 barrier filter and DF660 and DF630 interference filters. They were used to remove the background signal. Direct and lateral light scattering was analyzed. We studied not less than 10^4 cells in each sample.

RESULTS

Subpopulations of lymphocytes were identified by standard methods based on flow cytofluorometry [13]. We studied direct (angles 1-10°) and lateral light scat-

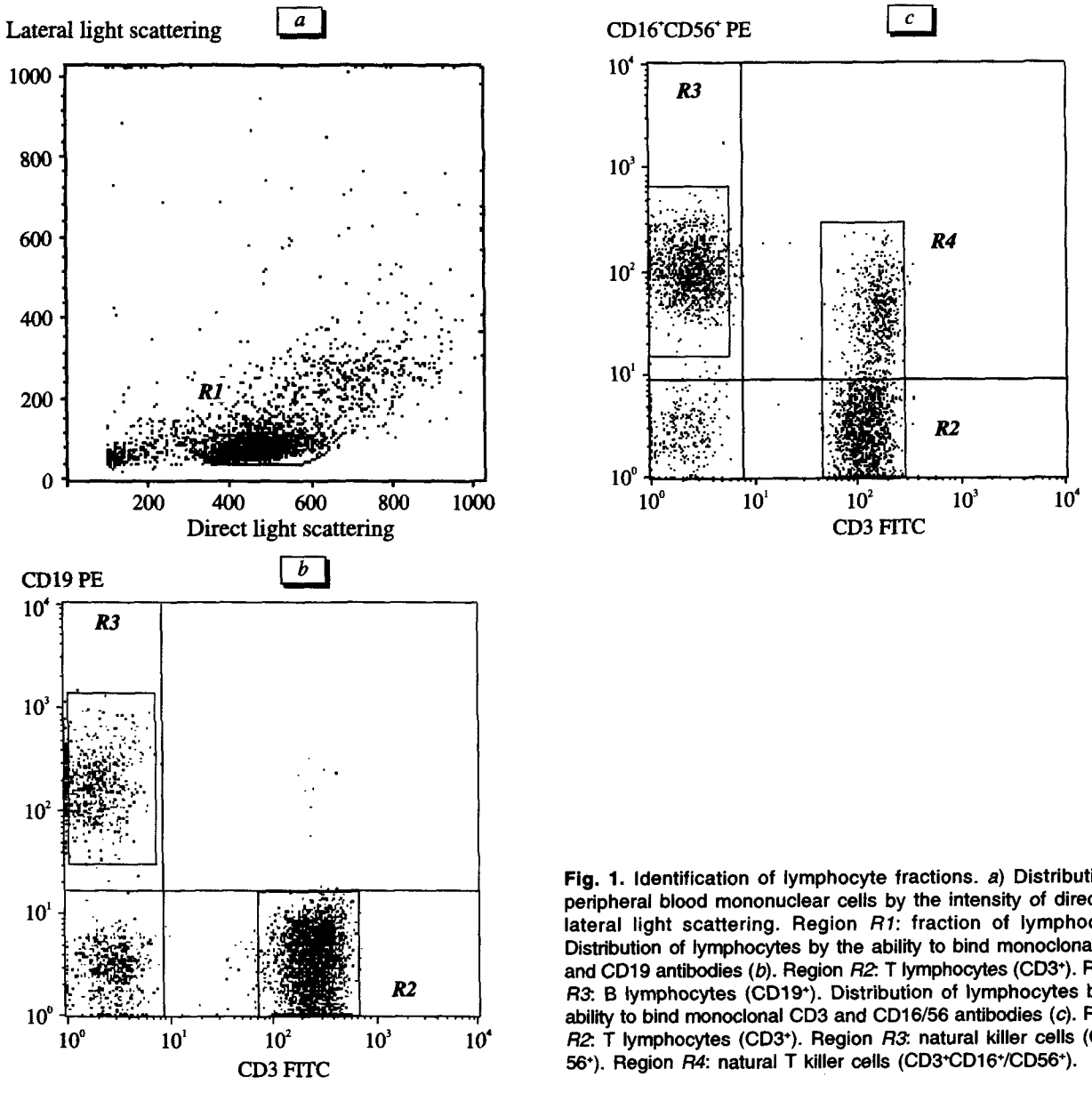

Fig. 1. Identification of lymphocyte fractions. *a*) Distribution of peripheral blood mononuclear cells by the intensity of direct and lateral light scattering. Region *R1*: fraction of lymphocytes. Distribution of lymphocytes by the ability to bind monoclonal CD3 and CD19 antibodies (*b*). Region *R2*: T lymphocytes ($CD3^+$). Region *R3*: B lymphocytes ($CD19^+$). Distribution of lymphocytes by the ability to bind monoclonal CD3 and CD16/56 antibodies (*c*). Region *R2*: T lymphocytes ($CD3^+$). Region *R3*: natural killer cells ($CD16/56^+$). Region *R4*: natural T killer cells ($CD3^+CD16^+/CD56^+$).

tering (angle 90°) that characterize the linear size and granularity of cells, respectively.

The isolated fraction of lymphocytes was characterized by low values of direct and lateral light scattering (Fig. 1, *a*, region *R1*). Monocytes and dead cells were excluded from the analysis. We identified T lymphocytes, B lymphocytes, and natural killer cells specifically binding monoclonal CD3, CD19, and CD16/CD56 antibodies, respectively (Fig. 1, *b*, *c*, regions *R2* and *R3*). Costaining of T cells with CD4, CD8, and CD16/CD56 antibodies allowed us to isolate various subpopulations of T lymphocytes, including T helper cells ($CD3^+CD4^+$), T suppressor cells ($CD4^+CD8^+$), and natural T killer cells ($CD3^+CD16/CD56^+$).

Derivatives of Cl e6 and HP fluoresced in visible light at 660-670 and 632 nm, respectively. The fluorescence quantum yield in ethanol was 15-20%. Accumulation of pigments in lymphocytes was accompanied by an increase in cell fluorescence in the specific emission band. Histograms for the distribution of cells were shifted to a high-fluorescence area (Fig. 2).

Fluorescence of HP and Cl e6 esters in lymphocytes was lower compared to nonesterified derivatives by an order of magnitude (Fig. 2).

Published data show that monomers of Cl e6 and its esters accumulated in cells have the same spectral and energy characteristics of fluorescence [2]. The intensity of fluorescence was estimated to compare intracellular accumulation of chlorines. Similar statement is true for HP and HP dimethyl ester. Our studies showed that esterification of lateral carboxylic groups in the molecule of HP has no effect on spectral characteristics of the pigment.

The method of cell identification allowed us to evaluate accumulation of pigments in individual lymphocyte subpopulations (Tables 1 and 2).

We revealed significant differences in accumulation of photosensitizers in different cells. The intensity of staining of B lymphocytes ($CD19^+$) and natural killer cells ($CD16^+/CD56^+$) was much higher compared to T lymphocytes ($CD3^+$).

The amount of photosensitizers accumulated in T helper cells ($CD4^+$) was lower than that detected in cytotoxic T lymphocytes ($CD8^+$). The difference in staining of $CD8^+$ and $CD4^+$ with chlorines was 1.25. High-intensity staining was observed in T lymphocytes expressing CD16/CD56 and HLA-DR activation markers. Variations in staining of lymphocytes with sensitizers are related to differences in the chemical structure. The intensity of staining with dimethyl ester was slightly higher. Accumulation of photosensitizers in B lymphocytes and natural killer cells differed from that in T lymphocytes: for Cl e6, by 1.25 and 1.29 times, respectively; for dimethyl ester, by 1.42 and 1.52 times, respectively. Study of hematoporphyrin accumulation produced similar results.

Previous experiments showed that peripheral blood leukocytes have different ability to accumulate sensitizers. The degree of staining of granulocytes and monocytes with Cl e6 derivatives is higher compared to lymphocytes. These differences are associated structural and morphological characteristics of leukocytes from various fractions. Porphyrin sensitizers are localized in cell membrane structures [1]. Relatively large volume of cells and intracellular organelles determine intensive accumulation of chlorines and hematoporphyrins [6].

Differences in accumulation of porphyrin photosensitizers in lymphocytes are also associated with structural peculiarities of these cells. Study of binding of fluorescent probes to cells showed that B lymphocytes differ from T cells by higher membrane poten-

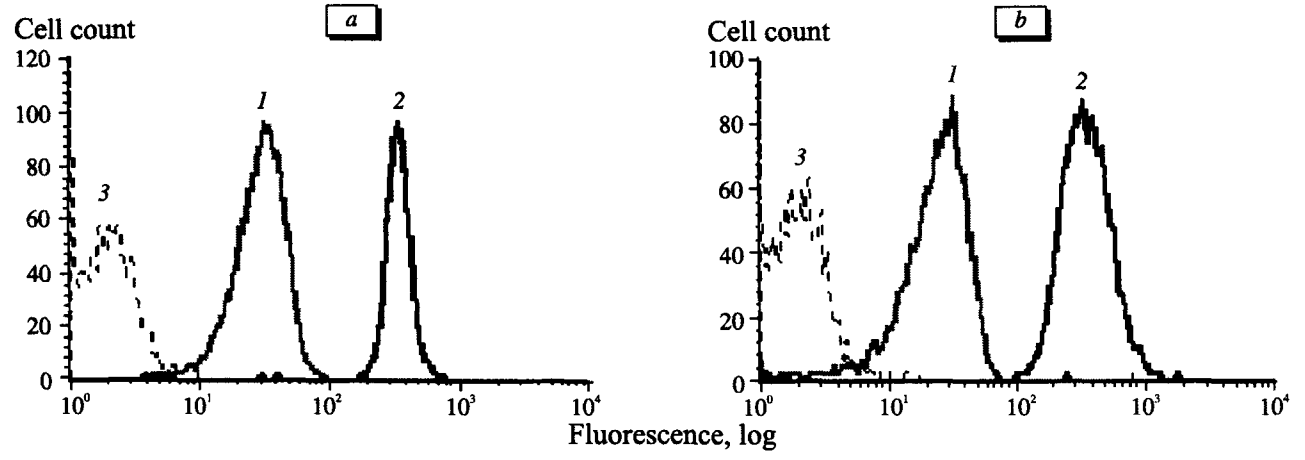

Fig. 2. Accumulation of chlorine e6 (Cl e6) and hematoporphyrin (HP) derivatives in peripheral blood lymphocytes. *a*) Histogram for the distribution of peripheral blood lymphocytes by fluorescence (660 nm) after incubation with Cl e6 (*1*) and Cl e6 dimethyl ester (*2*) or without pigment (*3*). *b*) Histogram for the distribution of peripheral blood lymphocytes by fluorescence (630 nm) after incubation with HP (*1*) and HP dimethyl ester (*2*) or without pigment (*3*). Pigment concentration 5×10^{-6} M. Incubation: 37°C, 120 min.

TABLE 1. Relative Accumulation of Cl e6 and HP Derivatives in Peripheral Blood Lymphocytes of Various Subpopulations (*M±m*)

Cells	Photosensitizers			
	Cl e6	Cl e6 dimethyl ester	HP	HP dimethyl ester
$CD19^+/CD3^+$ B lymphocytes	1.25±0.02	1.42±0.03	1.31±0.03	1.38±0.06
$CD16^+CD56^+/CD3^+$ natural killer cells	1.29±0.02	1.52±0.04	1.35±0.04	1.45±0.07

Note. Accumulation of pigments in T lymphocytes $CD3^+$ is taken as a unit. Here and in Table 2: results of 4 independent experiments.

TABLE 2. Relative Accumulation of Cl e6 and HP Derivatives in T Lymphocytes of Various Subpopulations (*M±m*)

Cells	Photosensitizers			
	Cl e6	Cl e6 dimethyl ester	HP	HP dimethyl ester
Cytotoxic T lymphocytes*, $CD3^+CD8^+/CD3^+CD4^+$	1.24±0.07	1.27±0.06	1.20±0.04	1.32±0.05
Natural T killer cells**, $CD3^+CD16^+CD56^+/CD3^+CD16^-CD56^-$	1.28±0.08	1.29±0.06	1.31±0.06	1.41±0.07
Activated T lymphocytes***, $CD3^+HLADR^+/CD3^+HLADR^-$	1.33±0.07	1.35±0.07	1.25±0.04	1.29±0.04

Note. One unit: *accumulation of pigments in T helper cells ($CD3^+CD8^+$ T lymphocytes); **accumulation of pigments in T killer cells ($CD3^+CD16^-CD56^-$ T lymphocytes); *** accumulation of pigments in resting cells ($CD3^-HLADR^-$ T lymphocytes).

tial (*i.e.*, greater area of the plasma and intracellular membrane). The number of binding sites for the fluorescent probe ANS on T lymphocytes is much lower than on B lymphocytes [3]. Fluorescent microscopy showed 1.8-2-fold differences in MBA fluorescence (membrane probe) in T and B lymphocytes [15].

The relative size of B lymphocytes and natural killer cells is higher than that of T lymphocytes. Besides this, activated cells have a greater relative size than nonactivated cells. These characteristics probably contribute to differences in the amount of photosensitizers accumulated in cells.

Significant differences were revealed in the concentration of Cl e6, HP, and their esters in cells. Pronounced accumulation of esterified porphyrin derivatives is related to high affinity for cell membranes and ability to cross the plasma membrane and accumulate in intracellular membranes [5,15].

Our results illustrate significant differences in the ability of natural killer cells, T lymphocytes, B lymphocytes, and subpopulations of T lymphocytes to accumulate porphyrin pigments. The photocytotoxic effect of porphyrin pigments depends on intracellular photosensitizer concentration. It can be hypothesized that under similar conditions of staining, these subpopulations of lymphocytes will have different sensitivity to visible light. Selective staining of activated T lymphocytes with porphyrin photosensitizers is of considerable importance in this respect. T lymphocytes are involved in the pathogenesis of various autoimmune disorders, including rheumatoid arthritis, multiple sclerosis, and lupus erythematous. Intensive accumulation of photosensitizers in activated cells produces functional changes in T cells (modification of expression of activation markers, inhibition of proliferation) and induces apoptotic death [9-11]. Better understanding of the mechanisms of photodynamic treatment of immunocompetent cells would allow us to develop new methods of immunocorrecting therapy.

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