Cytokine-Induced Differentiation and Proliferation of Human T Lymphocytes *In Vitro*: Effects of Interleukin 2 and Interleukin 6

V. V. Smol'nikova, A. V. Voznyuk, and M. P. Potapnev*

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The contents of $CD8^+$, $CD4^+CD8^+$, $CD3^+HLA\text{-}DR^+$, $CD8^+INF\text{-}\gamma^+$ T cells, and natural killers ($CD16^+56^+$) and NK/T cells ($CD16^+56^+CD3^+$) increase after 7-day culturing in the presence of interleukin-2.The number of apoptotic cells and cells in S-, and G_2+M phases of the cell cycle also increased. Interleukin-6 predominantly induced proliferation of $CD3^+HLA\text{-}DR^+$ T cells and G_2+M mitotic cells.

Key words: *interleukin-2*; *interleukin-6*; *human T lymphocytes*

Proliferation and differentiation of T lymphocytes can be initiated by antigens, superantigens, polyclonal activators, and cytokines [6], in particular, interleukin-2 (IL-2). IL-2 and other growing factors can also act as factors of lymphocyte survival, and this effect is not associated with induction of cell proliferation [3]. IL-6, usually related to inflammatory response, also affects T cells and natural killers (NK), although it is mostly considered as a costimulator of their proliferation [11]. The role of these cytokines in the differentiation of immature T cells and NK is thoroughly investigated [10]. However, little is known on their effect on differentiation of mature T cells. Our aim was to evaluate viability and subpopulations of human T lymphocytes *in vitro* stimulated with IL-2 and IL-6.

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMC) were isolated from heparin-stabilized blood of healthy donors (State Hemotransfusion Station, Ministry of Health of the Belorussia Republic) by centrifugation in a Ficoll-Verografin gradient (d=1.0775 g/cm^3) and washed with RPMI-1640 medium (Serva) containing 1% heat-inactivated human serum group IV (AB). Some of these cells were used for immunophenotyping. Other PBMC were cultured (in a concentration of 2×10^6/ml) with or without cytokines for 3 or 7 days in RPMI-1640 medium supplemented with 10% AB serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human recombinant IL-2 (Cetus, specific activity 3×10^6 MU/mg protein) and IL-6 (8×10^6 MU/mg protein, Institute of Extrapure Proteins, St. Petersburg) were added to the culture in final doses of 10, 100, and 1000 MU/ml. The specificity and activity of cytokines were verified on CTLL-2 and 7 TD.1 mice using IL-2 (86/504) and IL-6 (88/514) international standards.

Viability of freshly isolated and cultured for 3 and 7 days PBMC evaluated by trypan blue exclusion was more than 98%, and no less than 80 and 60%, respectively.

Immunophenotyping was carried out on a FACScan flow cytofluorometer (Becton Dickinson). Pretreatment with fluorochrome-labeled monoclonal antibodies (MAB) was performed according to manufacturer's recommendations. FITC-labeled anti-CD3, anti-CD4, ficoerythrin (FE)-labeled anti-CD8, anti-HLA-DR, anti-$CD16^+56$ (Becton Dickinson), and anti-

Institute of Hematology and Hemotransfusion, Ministry of Health of the Belorussia Republic; *Republical Research and Practical Center of Children Oncology and Hematology, Ministry of Health of the Belorussia Republic, Minsk. ***Address for correspondence:*** potapnev@mpcdog.belpak.minsk.by. Potapnev M. P.

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CD95 MAB (Medbiospectr, Moscow) monoclonal antibodies were used. Then the cells were treated with secondary antibodies (FITC-labeled goat anti-mouse IgG antibodies, Becton Dickinson). The data were analyzed using Lysis-II software; 5000 cells were processed in each test.

Interferon-γ (INF-γ) and IL-4 in $CD4^+$ and $CD8^+$ T cells were detected as described previously [7]. Briefly, the cells were preincubated with 2 mmol monensin for 4 h, treated with FITC-labeled anti-CD4 or anti-CD8 MAB, washed, and incubated with anti-INF-γ or anti-IL-4 MAB (Serva) in the presence of 0.1% saponin (Serva), washed again, and incubated with FE-labeled second antibodies. Immunofluorescence was measured on a FACScan cytofluorometer.

Phases of the cell cycle and apoptosis in culture of PBMC stained with propidium iodide (Serva) were analyzed by flow cytometry using CellFit software. Cells in the sub-G_1 peak of DNA histogram were considered as apoptotic [4].

The data were analyzed statistically by Student's *t* test. All parameters were evaluated in triplicates.

RESULTS

We previously showed that IL-6 in doses of 100 and 1000 MU/ml induced proliferation of human PBMC, which was detected after 7, but not after 3 days in culture (as for IL-2) [12]. We hypothesised that different dynamics of cytokine-induced PBMC proliferation was due to recruitment of different cell subpopulations. Since proliferating lymphocytes from *in vitro* culture were characterized by increased dispersion of light scattering, our measurements were performed in R2 area on the SSC/FSC plot characterized by fluorescence intensity above 400 arb. units on FSC scale and high specificity of MAB binding (Fig. 1). Area R1 reflected standard distribution of $CD4^+$ and $CD8^+$ T lymphocytes independent of culturing conditions. Area R2 included 10-30% viable cells in the 3-day culture and 35-55% viable cells in the 7-day culture. The contents of $CD19^+$ B lymphocytes and $CD14^+$ monocytes/macrophages were 5-7 and 2-4% of R2 area, respectively, irrespective of culturing conditions. It was found that phenotypic changes and other cell parameters directly correlated with cytokine concentration, the optimum concentration being 100 MU/ml. The distributions of T lymphocyte and NK subpopulations in initial PBMC in area R2 were similar to that of entire lymphocyte pool except for a minor increase in $CD8^+$ T cell and $CD16^+56^+$ NK populations (Table 1). Culturing with cytokines (100 MU/ml) for 3 days decreased the content of $CD3^+$ and $CD4^+$ T lymphocytes due to accumulation of $CD16^+56^+$ NK and $CD8^+$

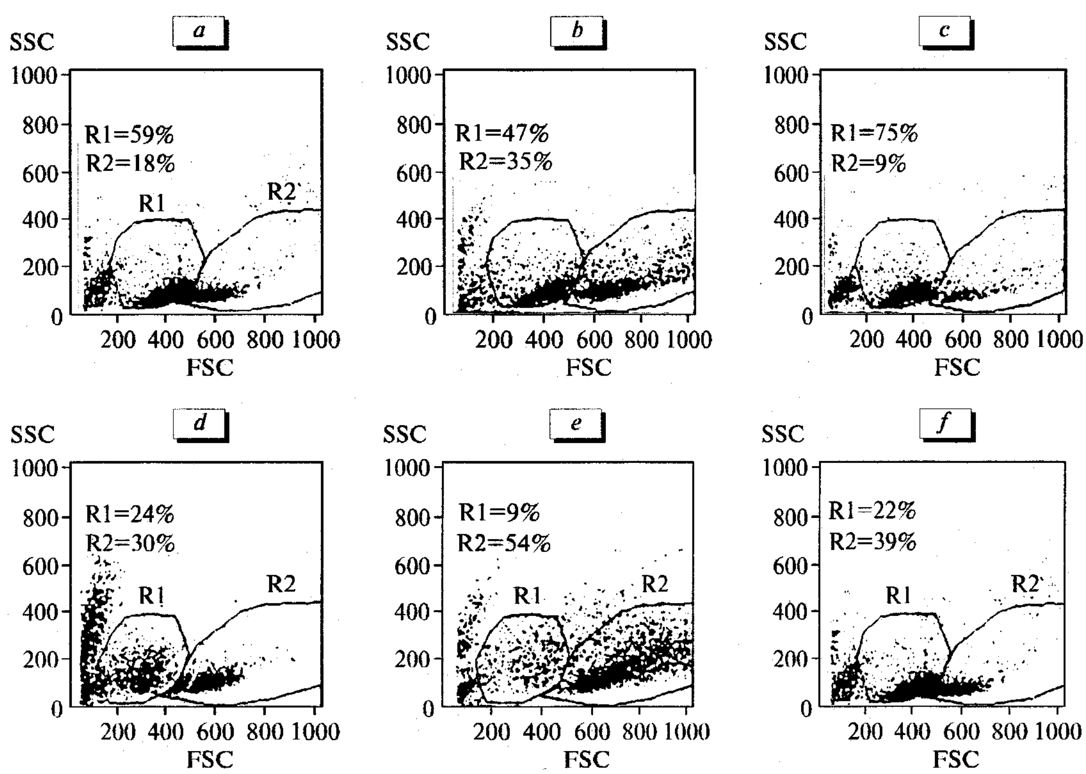

Fig. 1. Scatterogram of human lymphocytes cultured for 3 (*a*, *b*, *c*) and 7 days (*d*, *e*, *f*). FSC and SSC: forward and side light scattering. Nonactivated lymphocytes (*a*, *d*), lymphocytes activated with interleukin-2 (100 MU/ml, *b*, *e*) and interleukin-6 (100 MU/ml, *c*, *d*). Area R2 corresponds to proliferating lymphocytes. The plots present the results of a typical experiment (*n*=5).

TABLE 1. Subpopulations of Proliferating Lymphocytes (%) in Peripheral Mononuclear Cell Culture in the Presence of Human IL-2 and IL-6 (100 MU/ml, $M \pm m$, n=5)

Differentiation clusters	Baseline level	Time in culture, days					
		3			7		
		control	IL-2	IL-6	control	IL-2	IL-6
CD3	66.3±1.7	83.8±0.7	73.2±3.0*	70.6±4.4*	86.7±0.2	67.1±0.5*	80.1±0.3*
CD4	40.1±1.6	50.5±2.1	33.4±2.3*	39.2±6.6	53.6±6.3	33.9±9.4	53.9±8.7
CD8	31.9±2.5	40.0±2.1	47.8±3.6	51.6±7.2	39.2±4.7	57.9±4.6*	39.8±7.4
$CD3^+$HLA-DR	4.2±0.8	4.6±1.5	5.2±0.6	4.5±1.1	5.6±2.5	46.1±2.7*	24.9±0.3*
$CD16^+56$	18.6±2.2	11.2±2.7	23.2±2.3*	18.8±3.6	4.0±0.3	33.2±10.1*	7.3±1.5
$CD3^+CD16^+56$	3.9±0.7	4.6±1.4	5.2±0.6	4.5±1.1	2.0±0.5	13.9±6.2	2.6±1.2
$CD4^+CD8$	1.4±0.2	1.4±0.1	1.4±0.5	3.3±1.1	2.2±0.3	3.7±0.3*	3.4±0.2*

Note: Here and in Table 2, *$p<0.05$ compared to the corresponding control.

T cells. The observed changes were most pronounced in the presence of IL-2 and less expressed in the presence of IL-6. These IL-2-induced changes became more pronounced after 7 days in culture. In parallel, a great number of activated $CD3^+HLA\text{-}DR^+$ T cells appeared in culture and the content of mixed T/NK lymphocytes ($CD3^+CD16^+56^+$) and immature $CD4^+CD8^+$ T cells slightly increased. Culturing of PBMC with IL-6 (100 MU/ml) for 7 days significantly ($p<0.05$) increased the content of activated ($CD3^+HLA\text{-}DR^+$) and immature ($CD4^+CD8^+$) T lymphocytes against the background of decreased number of $CD3^+$ T cells and increased content of $CD16^+56^+$ NK. When the concentration of cytokines was reduced to 10 MU/ml, the

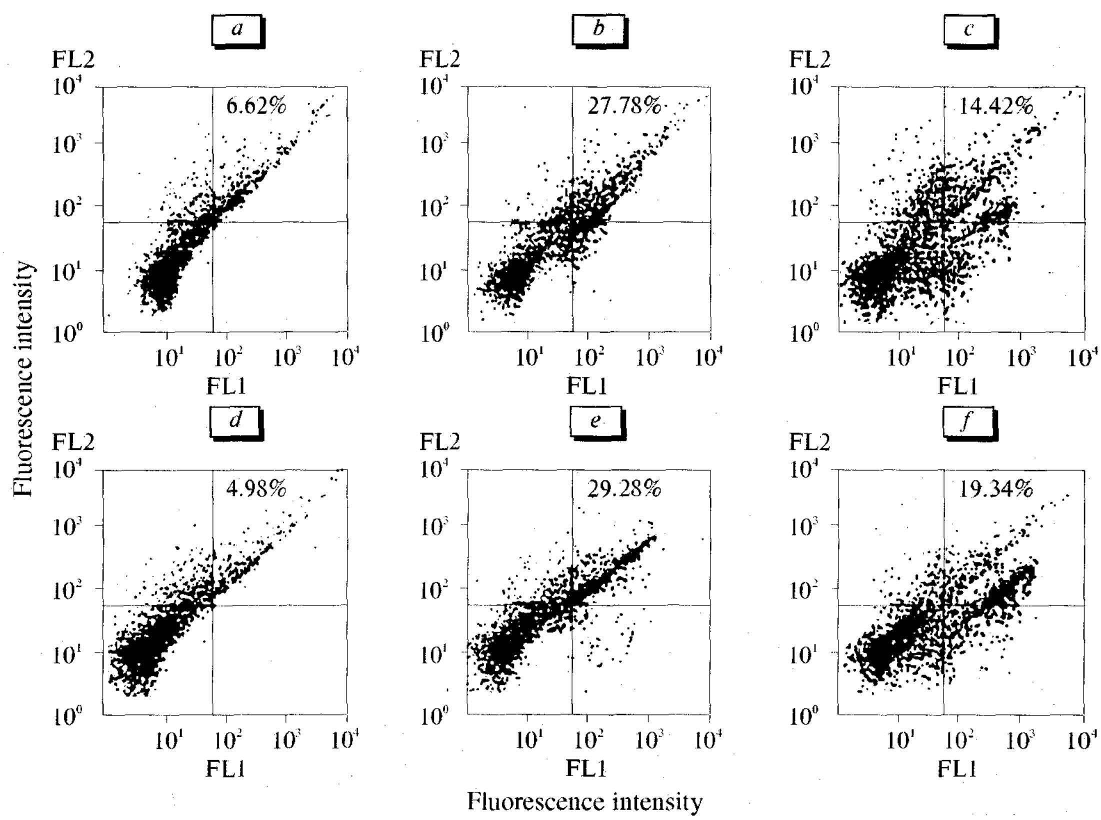

Fig. 2. Scatterplots of 7-day T lymphocyte culture secreting intracellular cytokines: $CD4^+$ T cells expressing interleukin-4 (IL-4) spontaneously (*b*) or in the presence of IL-6 (*e*); $CD8^+$ T cells expressing interferon-γ (INF-γ) spontaneously (*c*) or in the presence of IL-2 (*f*). Controls (*a*, *d*): cultured T lymphocytes treated with isotype-specific antibodies against CD4/CD8 or IL-4/INF-γ (FL2). The plots represent data of a typical experiment (n=6).

shifts in lymphocyte subpopulations became less significant. At higher doses of IL-2 (up to 1000 MU/ml), the distribution of lymphocytes in area R2 was similar to that induced by IL-2 in a dose of 100 MU/ml (data not shown).

We also studied the effect of IL-2 and IL-6 on Th1/Th2 differentiation of T cells. In 6 experiments the expression of intracellular cytokines (IL-4, INF-γ) by $CD4^+$ and $CD8^+$ T cells was assayed. IL-2 (100 MU/ml) increased the content of $CD8^+$ T cells expressing INF-γ (Fig. 2). IL-2 produced no significant effects on $CD4^+$ T cells producing INF-γ and on $CD4^+$ and $CD8^+$ T cells expressing IL-4 (data not shown). The differentiation of CD4+ T cells towards Th1 phenotype probably requires the presence of other cytokines apart from IL-2 [1]. IL-6 (100 MU/ml) had practically no effect on expression of INF-γ and L-4 by $CD4^+$ and $CD8^+$ T cells, though, in some experiments, the number of CD4+ T cells expressing IL-4 increased. Thus, IL-2 and IL-6 not only determine selection of T cell subpopulations, but also modulate cytokine production by these cells (IL-4 and INF-γ) [5].

Many cytokines maintain cell viability and proliferation and prevent their apoptotic death [3]. Culturing with IL-6 (100 MU/ml) for 7 days increased the number of proliferating lymphocytes in S- and G_2+M phases of the cell cycle (Table 2). In the presence of IL-2 (100 MU/ml), the number of S- and G_2+M lymphocytes sharply increased. Simultaneously, the number of aneuploid cells (sub-G1-peak on the DNA histogram) increased 2-fold. IL-6 did not enhanced apoptosis. Interestingly, the number of aneuploid cells did not depend on the number of Fas/$CD95^+$ lymphocytes in PBMC culture, probably because of the fact that expression of Fas/$CD95^+$ preceded apoptosis, while cells with aneuploidy were at the terminal state of apoptotic death [4]. In our experiments, the numbers of $CD95^+$ and aneuploid cells in area R2 reflected their contents in the entire population of viable lymphocytes (data not shown).

Thus, IL-2 exhibits a more potent proliferative activity *in vitro* with respect to human T- lymphocytes compared to IL-6, and significantly modulates proliferation of lymphocyte subpopulation, cytokines secretion, and induction of apoptosis.

TABLE 2. Distribution of Mitotic Phases in Proliferating Lymphocytes (%, *M*±*m*, *n*=4)

Mitotic phase	Non-activated (control)	Activated with IL-2 (100 MU/ml)	Activated with IL-6 (100 MU/ml)
G_0/G_1	96.1±1.9	84.3±2.0*	92.5±2.2
S	2.4±1.5	15.8±3.1*	6.2±2.0
G_2+M	0.4±0.1	3.0±0.8*	1.4±0.1*
Суб-G_1-peak	8.4±1.2	15.3±1.2*	9.1±1.9
$CD95^+$	78.2±2.5	24.9±6.5*	61.0±7.5

Note: $CD95^+$: number of cells expressing Fas/CD95.

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