Studying the Proliferation of Human Peripheral Blood T Lymphocytes in Serum-Free Medium

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We compared the cultivation of human peripheral blood lymphocytes in serum-free medium Hybris-2 and RPMI 1640 medium with 10% fetal bovine serum in the presence of phytohemagglutinin and interleukin-2. The optimal concentration of phytohemagglutinin significantly differed in serum-free and serum-containing media (0.5 and 5 mg/ml, respectively). Both mitogens were more potent in stimulating the proliferation of lymphocytes in serum-free medium than in serum-containing medium. Strong proliferation of $CD3^+$ and $CD4^+$ T lymphocytes was observed in both media. The dynamics of other markers was similar in serum-free and serum-containing media. However, significant differences were revealed between individual donors. Our results indicate that the developed serum-free medium may be used in lymphocyte cultivation for scientific, diagnostic, and therapeutic purposes.

Key Words: *serum-free medium; mononuclear leukocytes; T lymphocytes; phytohemagglutinin; interleukin-2*

Much progress in clinical diagnostics, cell therapy, and biotechnology necessitates the use of high-efficacy serum-free media for cultivation of human cells [7,8]. Peripheral blood lymphocytes are extensively used for these purposes. It is associated with the simplicity of isolation and high potency of these cells in the diagnostics, therapy, and biotechnology. The methods for large-scale cultivation of lymphocytes are of particular importance, which results from the development of cellular immunotherapy for human diseases.

Lymphocytes are usually cultured in RPMI 1640 medium enriched with 10-20% fetal bovine serum (FBS) or bovine serum [2,3]. These sera differ in the qualitative and quantitative composition of factors, which can inhibit or stimulate lymphocyte proliferation. Therefore, serum-free media hold much promise for cell cultivation [14]. In the presence of blood plasma, a variety of growth factors and other biologically active substances exist in the bound form [6]. Hence, the interaction of these compounds with cells significantly differs in the presence and absence of blood plasma. This fact should be taken into account in the development of serum-free medium and interpretation of data on the interaction of substances with the cell in serum-containing media.

We developed the serum-free medium Hybris-2, which maintains the growth of hybridoma cells. However, peripheral blood lymphocytes could not be cultured in this medium under standard conditions.

This work was designed to study the cultivation of peripheral blood lymphocytes in serum-free media. Here we optimized the content of mitogens in these media.

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MATERIALS AND METHODS

Experiments were performed with the following media and reagents (PanEco): phytohemagglutinin P (PHA), nutrient medium RPMI 1640 with glutamine; serum-free medium Hybris-2; Ficoll solution (d= 1.077 g/ml); Hanks solution not containing Ca^{2+}, Mg^{2+}, and phenol red; FBS (K055, PAA Laboratories); and recombinant interleukin-2 (rhIL-2, PAN-Biotech).

Fresh heparinized blood (50 U/ml) was sampled from two healthy donors of reproductive age, which differed in the proliferative response of mononuclear cells (MNC) to stimulation with PHA in serum-containing medium (RPMI 1640+FBS 10%) and serum-free medium (Hybris-2).

The mononuclear fraction of leukocytes was obtained by the standard method using a one-step Ficoll gradient (d=1.077 g/ml). Fresh heparinized blood (20 ml, 50 U/ml) was obtained from the donor, diluted by Hanks solution without Ca^{2+} and Mg^{2+} (by 2 times), and layered on Ficoll solution (d=1.077 g/ml, ratio 3:1). The diluted blood was centrifuged at 400*g* and room temperature for 30 min. Interphase cells were washed 2 times by Hanks solution without Ca^{2+} and Mg^{2+} and resuspended in RPMI 1640 medium. The cells were counted and used for cultivation.

The optimal concentration of PHA in serum-containing and serum-free media was estimated. The mononuclear fraction of leukocytes (2.0×10^5 cells) was resuspended in 1 ml serum-containing medium (RPMI 1640+FBS 10%) and serum-free medium (Hybris-2) with PHA at various concentrations. The treatment was performed using 24-well culture plates. Further incubation was conducted in a CO_2 incubator at 37°C and 5% CO_2. A microscopy study and cell counting in a Goryaev chamber were performed 120 h after the start of incubation to evaluate the degree of stimulation of the lymphocyte proliferative response in serum-containing and serum-free media.

A comparative study was performed to evaluate the dynamics of changes in lymphocyte count. The mononuclear fraction of leukocytes (4.5×10^5 cells) was resuspended in 1 ml nutrient media, which contained PHA at concentrations of 0.5 (Hybris-2) or 5.0 mg/ml (RPMI 1640+FBS 10%). Incubation was performed in 24-well culture plates using a CO_2 incubator (37°C, 5% CO_2). A microscopy study and cell counting (4 samples for each medium) were performed at 24-h intervals (0-168 h) to evaluate the effectiveness and characteristics of MNC cultivation in serum-containing and serum-free media.

The expression of specific markers for MNC subpopulations (CD3, CD4, CD8, CD16, CD19, and HLA-DR) during cultivation in serum-containing and serum-free media was evaluated 0, 72, and 120 h after the start of incubation. The measurement were performed on a FACSCalibur flow cytofluorometer (Becton Dickinson) using FITC-labeled mononuclear antibodies to the corresponding antigens. The results were analyzed by WINMDI 2.8 software.

T lymphocyte cultivation in the presence of IL-2 was performed in two stages. In stage I, the isolated MNC were stimulated with PHA in serum-containing (5.0 mg/ml) and serum-free media (0.5 mg/ml) for 72 h. In stage II, the stimulated cells were collected by centrifugation, washed with Hanks solution not containing Ca^{2+} and Mg^{2+}, and resuspended in the corresponding media to a concentration of 10^6 cells/ml. The cells (10^5 cells/ml) were put in wells of a culture plate with serum-containing and serum-free media. The concentration of IL-2 was 0, 5, 10, 25, 50, or 100 ng/ml. The density of cell suspensions was measured. The number of cells in a Goryaev chamber was estimated 72-192 h after the start of incubation.

RESULTS

The induction of lymphocyte proliferation was studied in the presence of PHA at various concentrations. Fig. 1 shows the optimal concentrations of PHA to induce proliferation of isolated lymphocytes in serum-containing medium (RPMI 1640+FBS 10%) and serum-free medium (Hybris-2). The optimal induction concentration of PHA in serum-free medium was 6-10 times lower than in serum-containing medium. A narrow range of the optimum in serum-free medium was close to 0.5 mg/ml. The optimal induction concentration of PHA in serum-containing medium was 3.0-6.0 mg/ml. The distribution curve had a more smooth profile under these conditions. A smaller stimulatory effect of PHA in serum-containing medium is probably related to mitogen inactivation by serum components. Published data show that PHA binds to certain fractions of serum globulins [4,5]. The phenomenon of binding and transport functions of blood components is well known in pharmacology and physiology [1]. For example, the effect of biologically active substances on cells in serum-containing media often depends on the modulatory action of serum components. Therefore, the use of serum-free media holds much promise to study a direct effect of test substances and agents on target cells.

The quantitative dynamics of lymphocyte proliferation in serum-containing and serum-free media was studied for 7 days in the absence and presence

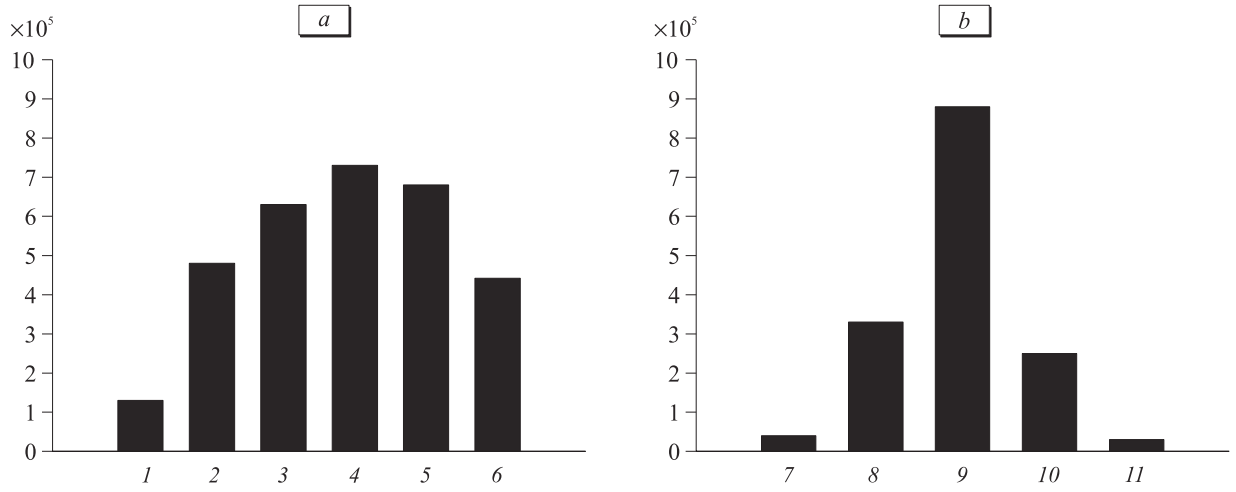

Fig. 1. Dependence of lymphocyte number on PHA concentration in the medium 120 h after the start of incubation. Serum-containing medium (RPMI 1640+10% FBS, *a*); serum-free medium Hybris-2 (*b*). Concentration in serum-containing medium (mg/ml): control (*1*), 0.5 (*2*), 1.0 (*3*), 3.0 (*4*), 6.0 (*5*), 12.0 (*6*). Concentration in serum-free medium (mg/ml): control (*7*), 0.2 (*8*), 0.5 (*9*), 1.0 (*10*), 3.0 (*11*).

of PHA. Fig. 2, *a* shows a decrease in the number of MNC from donor 1 during incubation in serum-containing and serum-free media without PHA. MNC number in serum-free medium Hybris-2 decreased by 20% over the first 24 h of cultivation, but remained practically unchanged in RMPI 1640+10% FBS. It may be suggested that serum factors *in vitro* maintain viability of the primary MNC culture.

Fig. 2, *b*, *c* shows the number of PHA-stimulated MNC from donors 1 and 2 in serum-containing and serum-free media. Previous studies revealed that the number of MNC decreases during cocultivation with mitogens for 24-48 h, but increases by the 72nd hour of incubation [10,12]. The number of PHA-stimulated MNC from both donors increased more rapidly in serum-free medium than in serum-containing medium. Proliferation of MNC from donor 1 was characterized by a 2-fold increase in cell number in serum-free medium Hybris-2 (as compared to serum-containing medium RPMI 1640+10% FBS). This parameter in donor 2 was 1.2-fold higher than in donor 1.

TABLE 1. Dynamics of Changes (Percent Expression) in Lymphocyte Subpopulations during Cultivation in Serum-Free Medium Hybris-2 and RPMI 1640 Medium with 10% FBS

Medium; donor, time		Marker					
		CD3	CD4	CD8	CD16	CD19	HLA-DR
RPMI 1640+10% FBS							
donor 1	0 h	66.3	37.2	29.8	62.5	51.7	7.1
	72 h	93.1	50.6	42.0	2.4	0.6	32.5
	120 h	93.0	93.2	0.3	1.6	11.1	17.0
donor 2	0 h	55.4	26.9	28.9	12.0	11.0	46.3
	72 h	30.1	3.4	27.1	0.6	51.9	1.3
	120 h	97.9	15.8	83.5	2.2	0.7	65.5
Hybris-2							
donor 1	0 h	66.3	37.2	29.8	62.5	51.7	7.1
	72 h	70.9	29.8	39.7	0.4	2.7	25.5
	120 h	75.0	68.0	7.1	9.2	6.9	24.2
donor 2	0 h	55.4	26.9	28.9	12.0	11.0	46.3
	72 h	72.2	1.2	70.5	3.0	36.2	2.9
	120 h	98.6	9.5	88.8	90.9	2.1	22.4

Note. Data for two donors.

Human peripheral blood lymphocytes constitute a heterogeneous cell population. It was important to evaluate the subclasses of lymphocytes that contribute to the observed changes in cell populations. Cultured cells were characterized by the presence of surface markers (CD3, CD4, CD8, CD16, CD19, and HLA-DR; Table 1).

The dynamics of variations in subpopulations of PHA-stimulated lymphocytes was similar in serum-containing and serum-free media. We revealed an increase in the number of lymphoid T cells ($CD3^+$, up to 90%) and subpopulation of T helper cells ($CD4^+$, by 1.5-2 times). Lymphocyte subpopulations with other markers were shown to decrease during cultivation. Significant changes were revealed between various donors.

PHA-stimulated MNC were cultured with IL-2 at various concentrations (0, 5, 10, 25, 50, and 100 ng/ml) for 192 h. The cytokine IL-2 was a potent growth stimulator of PHA-stimulated T lymphocytes in serum-containing and serum-free media (Fig. 3, *a-d*). The induction with PHA for 72 h was insufficient to maintain cell proliferation in mitogen-free media. The optimal concentration of IL-2 did not differ in serum-containing and serum-free media. Studying the dynamics of lymphocyte proliferation showed that the optimal concentration of IL-2 is shifted from the low value to the high concentration of the cytokine, which depends on the time of cell cultivation with IL-2. It was probably related to the utilization of excess IL-2, which inhibits cell growth [13]. The effective concentration of free IL in serum-containing medium progressively decreases under these conditions. The rate of utilization may be reduced due to the ability of serum proteins (*e.g.*, α_2-macroglobulin) to bind reversibly IL-2 [9,11]. This hypothesis is confirmed by the fact that lymphocyte proliferation in Hybris-2 rapidly decreases at low doses of the cytokine and long-term incubation (Fig. 3, *c*, *d*). Despite the absence of differences between the optimal concentrations of IL-2 in serum-containing and serum-free media, Hybris-2 medium was shown to maintain a higher density of cultured lymphocytes at an IL-2 concentration of 50-100 ng/ml (by 10-120%).

Our experiments were performed with two mitogens. The interaction of these mitogens with serum proteins was described previously [4,5,9,11]. The stimulating agents had various effects on lymphocyte proliferation in serum-containing and serum-free media. These data illustrate differences in the interaction of mitogens with cells and/or binding proteins. This approach holds promise to study the

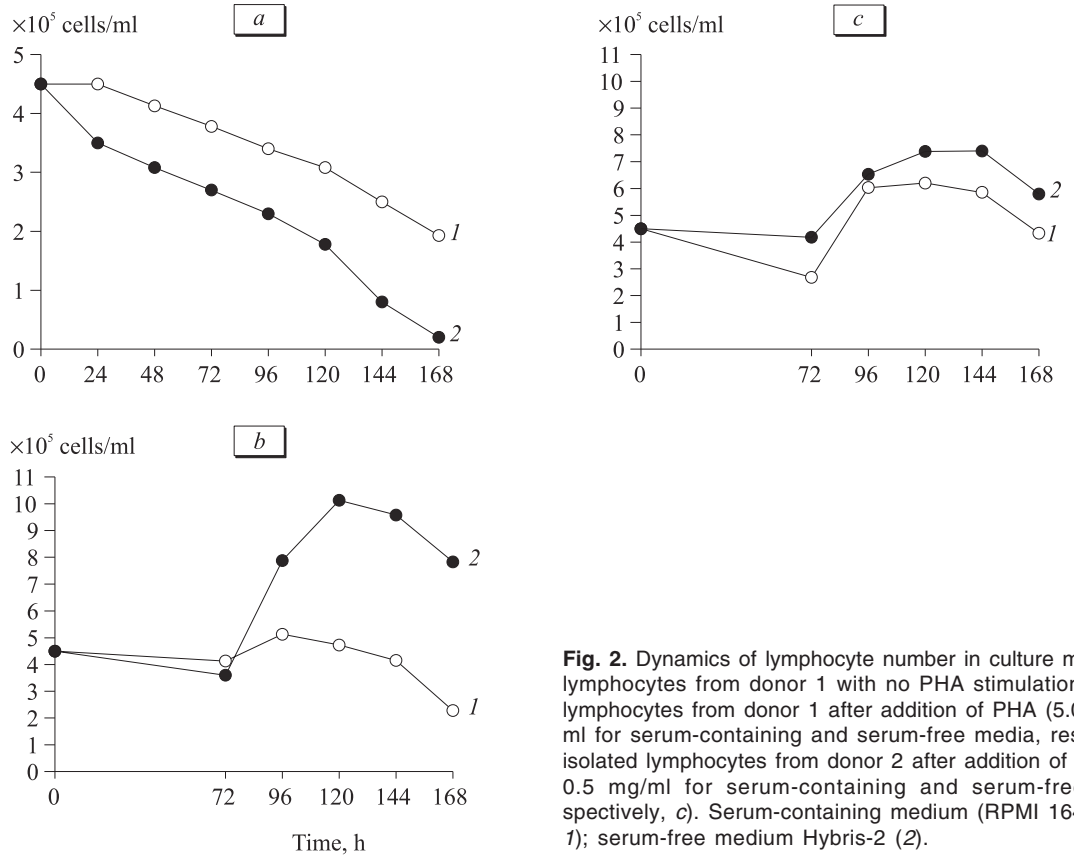

Fig. 2. Dynamics of lymphocyte number in culture media. Isolated lymphocytes from donor 1 with no PHA stimulation (*a*); isolated lymphocytes from donor 1 after addition of PHA (5.0 and 0.5 mg/ml for serum-containing and serum-free media, respectively, *b*); isolated lymphocytes from donor 2 after addition of PHA (5.0 and 0.5 mg/ml for serum-containing and serum-free media, respectively, *c*). Serum-containing medium (RPMI 1640+10% FBS, *1*); serum-free medium Hybris-2 (*2*).

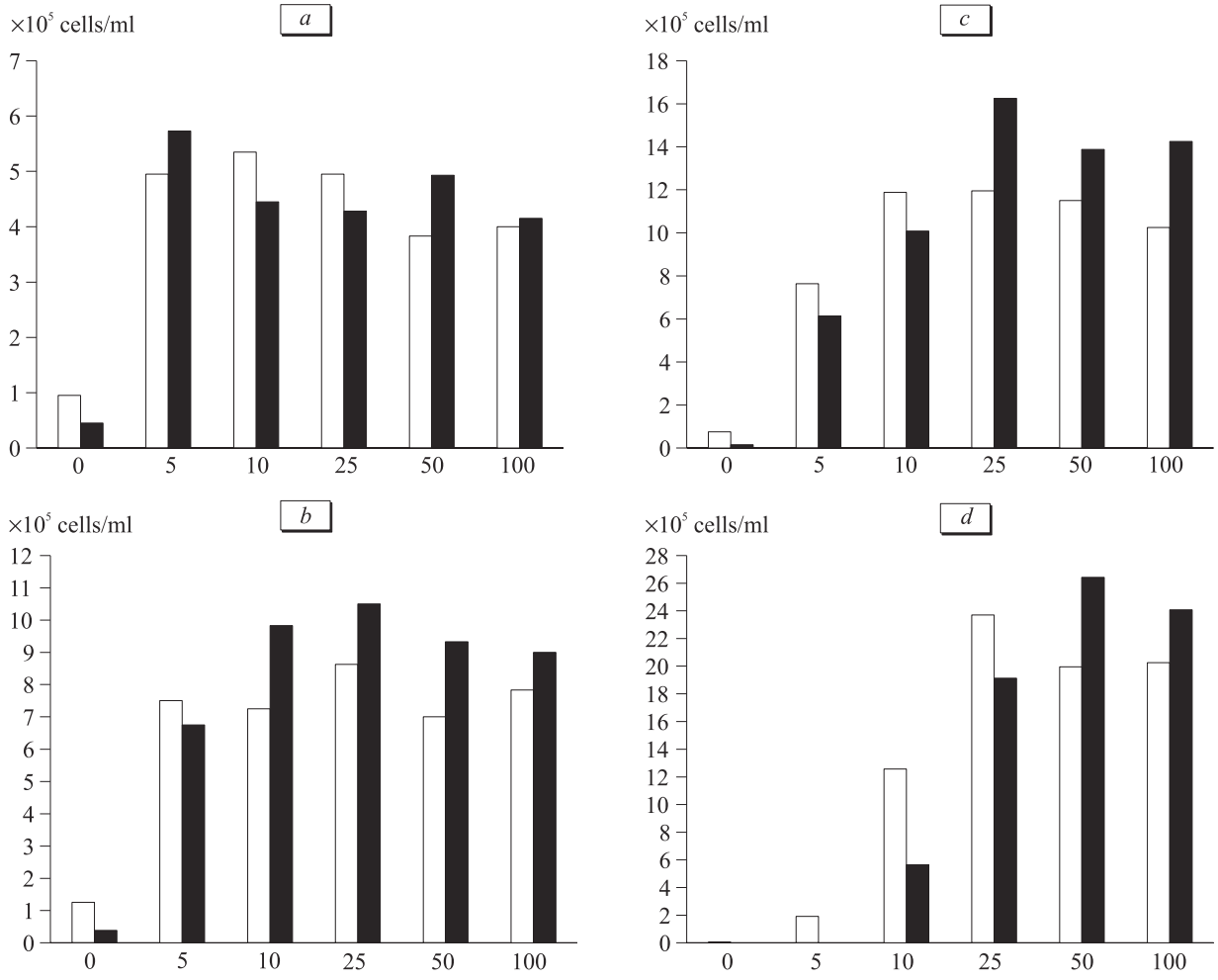

Fig. 3. Dependence of lymphocyte number on IL-2 concentration in culture media. After 72 h (*a*); after 96 h (*b*); after 120 h (*c*); after 192 h (*d*). Light bars, serum-containing medium (RPMI 1640+10% FBS); dark bars, serum-free medium Hybris-2.

interaction of various biologically active substances with the cell.

We conclude that the developed serum-free medium is potent in maintaining the proliferation of lymphocytes.

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